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(54) Title: RECOMBIANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS, CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF			
(57) Abstract <p>An adenovirus E1/E4 expressing packaging cell line is provided, which permits the generation of recombinant adenoviruses deleted in both gene regions. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell is provided by infecting a target cell with a recombinant AAV comprising a selected transgene under the control of regulatory sequences. The infected cell is contacted with an agent which facilitates the conversion of single stranded recombinant virus to its double stranded form.</p>			

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RECOMBINANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS,
CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF

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Institute of Health Grant Nos. HD32649-01, DK47757 and
5 DK49136. The United States government has rights in this
invention.

Field of the Invention

The present invention relates generally to the field
of somatic gene therapy, and specifically to methods and
10 compositions useful in the treatment of genetic
disorders.

Background of the Invention

Adenoviruses are eukaryotic DNA viruses that can be
modified to efficiently deliver a therapeutic or reporter
15 transgene to a variety of cell types [see, e.g., M. S.
Horwitz et al, "Adenoviridae and Their Replication",
Virology, second edition, pp. 1712, ed. B. N. Fields et
al, Raven Press Ltd., New York (1990)]. Recombinant
adenoviruses (rAds) are capable of providing extremely
20 high levels of transgene delivery to virtually all cell
types, regardless of the mitotic state. The efficacy of
this system in delivering a therapeutic transgene *in vivo*
that complements a genetic imbalance has been
demonstrated in animal models of various disorders [K. F.
25 Kozarsky et al, Somatic Cell Mol. Genet., 19:449-458
(1993) ("Kozarsky et al I"); K. F. Kozarsky et al, J.
Biol. Chem., 269:13695-13702 (1994) ("Kozarsky et al II")
and others]. The use of recombinant adenoviruses in the
transduction of genes into hepatocytes *in vivo* has
30 previously been demonstrated in rodents and rabbits [see,
e.g., Kozarsky II, cited above, and S. Ishibashi et al,
J. Clin. Invest., 92:883-893 (1993)].

The first-generation recombinant, replication-
deficient adenoviruses which have been developed for gene
35 therapy contain deletions of the entire E1a and part of
the E1b regions. This replication-defective virus is

grown on an adenovirus-transformed, complementation human embryonic kidney cell line containing a functional adenovirus E1a gene which provides a transacting E1a protein, the 293 cell [ATCC CRL1573]. E1-deleted viruses
5 are capable of replicating and producing infectious virus in the 293 cells, which provide E1a and E1b region gene products ~~in-trans~~. The resulting virus is capable of infecting many cell types and can express the introduced gene (providing it carries its own promoter), but cannot
10 replicate in a cell that does not carry the E1 region DNA unless the cell is infected at a very high multiplicity of infection.

Adeno-associated virus (AAV) is an integrating human DNA parvovirus which has been proposed for use as a gene
15 delivery vehicle for somatic gene therapy [B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. This small non-enveloped virus contains a 4.6 kb single stranded (ss) DNA genome that encodes sets of regulatory and capsid genes called
20 *rep* and *cap*. *Rep* polypeptides (*rep78*, *rep68*, *rep62* and *rep40*) are involved in replication, rescue and integration of the AAV genome. The *cap* proteins (VP1, VP2 and VP3) form the virion capsid. Flanking the *rep* and *cap* open reading frames at the 5' and 3' ends are 145
25 bp inverted terminal repeats (ITRs), the first 125 bp of which are capable of forming Y- or T-shaped duplex structures.

Recombinant forms of AAV (rAAV) have been developed as vectors by replacing all viral open reading frames
30 with a therapeutic minigene, while retaining the necessary *cis* elements contained in the ITRs. [See, e.g., US Patent Nos. 4,797,368; 5,153,414; 5,139,941; 5,252,479; and 5,354,678; and International Publication Nos. WO 91/18088 published November 28, 1991; WO 93/24641
35 published December 9, 1993 and WO94/13788 published June

23, 1994]. However, progress towards establishing AAV as a transducing vehicle for gene therapy has been slow for a variety of reasons. For example, the integrated provirus preferentially targets specific sites in chromosome 19. Additionally, difficulties surround large-scale production of replication defective recombinants. The cells employed to produce rAAV must also be infected with adenovirus or herpesvirus to provide the necessary helper functions, thereby producing problems in purifying recombinant AAV (rAAV) from contaminating virus in culture. Practical experience with purified recombinant AAV as a gene therapy vector has been disappointing, because the more purified the AAV is from co-infection with its helper virus in culture, the lower the gene transduction efficiencies that the rAAV displays.

There remains a need in the art for additional recombinant adenoviruses and rAAV, therapeutic compositions and methods which enable effective use of these recombinant viruses in the treatment of disorders and diseases by gene therapy.

Summary of the Invention

In one aspect of this invention, a packaging cell line is provided which expresses adenovirus genes E1a, E1b and E4, or functional fragments thereof, e.g., the E4 open reading frame (ORF) 6.

In another aspect, the invention provides a rAd comprising the DNA of at least a portion of the genome of an adenovirus having functional deletions of the E1 and E4 gene regions; a suitable gene operatively linked to regulatory sequences directing its expression, and an adenovirus capsid, the rAd capable of infecting a mammalian cell and expressing the gene product in the cell *in vivo* or *in vitro*. The invention also provides a mammalian cell infected with the rAd described above.

In still another aspect, the invention provides a rAd shuttle vector comprising the DNA of at least a portion of the genome of an adenovirus having functional deletions of the E1 and E4 gene regions.

5 In a further aspect, the invention provides a method for producing the above-described recombinant Ad and a ~~method for delivering a selected gene into a mammalian~~ cell using the recombinant Ad described above.

10 In another aspect, the invention provides a method for enhancing the efficiency of transduction of a recombinant AAV into a target cell. The method operates, in brief, by infecting a target cell with a ss recombinant adeno-associated virus (rAAV) which comprises a transgene operatively linked to regulatory sequences
15 directing its expression, and contacting the infected cells with an agent which facilitates the conversion of ss rAAV to its double stranded (ds) form. Conversion of ss rAAV to ds rAAV occurs in the target cell, resulting in enhanced transduction of the rAAV into the target
20 cell. The agent may be a helper virus which carries a selected gene or functional fragment thereof encoding a polypeptide capable of enhancing the conversion of the ss rAAV to ds rAAV and which is co-infected into the same target cell. The agent may also be a drug or chemical
25 composition which accomplishes the same function and is applied to the infected target cell. This method can operate both in an ex vivo setting and in vivo.

In yet another aspect, the invention provides a novel recombinant AAV, which contains both the transgene
30 intended for use in treating a genetic disease or disorder and at least one additional gene operatively linked to inducible or constitutive regulatory sequences. The additional gene(s) encodes a polypeptide capable of facilitating, alone or in concert with other additional
35 genes, the conversion of ss rAAV to its ds form upon

expression. In a preferred embodiment, the additional gene is adenovirus E4 or a functional fragment thereof. Also disclosed is a method for enhancing the efficiency of transduction of the novel rAAV into a target cell.

5 The novel rAAV and methods of this invention are also useful in pharmaceutical compositions for use in ~~ex vivo and in vivo~~ gene therapy treatment protocols for treating inherited diseases, cancer, and other genetic dysfunctions.

10 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

15 Fig. 1 is a schematic drawing of an exemplary plasmid pMMTVE4ORF6 [SEQ ID NO: 1] or pMTE4ORF6, which contains an MMTV or sheep MT promoter, respectively, in control of a human E4 ORF 6 gene sequence, a growth hormone gene terminator sequence (GH), an SV40 ori, pBR322-based plasmid sequences including a neo^R gene, an
20 SV40 polyA site and an amp^R gene.

25 Fig. 2 is a schematic map of rAd H5.001CBLacZ [SEQ ID NO: 3] with indicated restriction endonuclease enzyme sites. The striated bar represents the CBLacZ minigene; the black bar represents Ad5 viral backbone, the crosshatched bar represents Ad E4 deletion.

30 Fig. 3 plots LacZ forming units (LFU)/ml vs time (hours) for E4 complementing cell lines infected with H5.001CBLacZ.

35 Fig. 4A is a graph of the induction, ORF6 expression and viral production in 293-27-18 packaging cells plotting yield at 24 hours post-infection (pi) in LFU/ml and ORF6 protein (abs.mm) vs. concentration of the inducer, dexamethasone (μ M). Abs.mm is the intensity of the size of the protein band on a Western blot and reflects absorbance and protein size in mm². The square

is yield at 24 hours pi. The diamond is ORF6 protein detected at 24 hours pi.

Fig. 4B is a similar graph to that of Fig. 4A, except that the packaging cells are 293-10-3 cells. The
5 symbols are as described for Fig. 4A.

Fig. 5A is a bar graph plotting β -galactosidase enzyme activity in lysates from infected HeLa cells. The
horizontal axis indicates the adenoviruses infected into
the HeLa cells, with the symbol "+" indicating the
10 addition of the adenovirus to the rAAV, AV.CMV β LacZ. The
vertical axis indicates intracellular β -galactosidase
specific activity (mUnits/mg protein) using ONPG. Below
each bar, the fold-induction in specific activity
relative to cells that received the AV.CMV β LacZ vector
15 alone is given.

Fig. 5B is a bar graph plotting Ad multiplicity of infection (MOI) in HeLa cells of wild-type Ad5 or the E2
mutant dl802, the cells co-infected with rAAV vs.
intracellular β -galactosidase specific activity. See
20 Example 11.

Fig. 6A is a graph in which β -galactosidase specific activity and counts per minute (CPM) are plotted along
the vertical axis and adenovirus MOI's are on the
horizontal axis for HeLa cells infected with wtAd5 and
25 rAAV according to Example 12. Data obtained from low MOI
(1, 5, and 10) infections are shown.

Fig. 6B is a graph similar to that of Fig. 6A except
that the cells were infected with Ad mutant dl802.

Fig. 7A illustrates a model for leading strand
30 synthesis of a complementary AAV strand in the presence
of Rep (+Rep) or absence of Rep (-Rep). Rep expresses a
terminal resolution activity that can convert a duplex
structure with closed-ends to an open-ended duplex. In
the absence of Rep, terminal resolution is impaired
35 leaving the covalently closed, hairpin structures intact.

Under these conditions, hairpins are expected to be found leftward and rightward, since both strands of a rescued ds AAV genome are packaged into virions.

Fig. 7B is a schematic of linear AV.CMV~~LacZ~~ with labeled domains including the AAV ITRs, CMV immediate early enhancer/promoter (CMV), SV40 splice donor-splice acceptor (SD/SA), ~~E. coli β -galactosidase cDNA (LacZ)~~, and SV40 polyA signal (pA). Two NotI sites located at bp positions 1035 and 4509 are indicated.

Fig. 7C illustrates a closed end and an open end fragment of rAV.CMV~~LacZ~~.

Figs. 7D, 7E and 7F indicate the mixture of open-ended and covalently closed duplex fragments generated by NotI digestion of ss AV.CMV~~LacZ~~ at position 4509 in the absence of terminal resolution. The NotI 4509 digestion provides a convenient means of releasing a 361 bp fragment that contains the right ITR in the context of a hybridization target (i.e. SV40 pA). In the presence of terminal resolution, only the open-ended 361 bp fragment would be expected to be generated (Fig. 7D) by such digestion.

Fig. 8A is a bar graph plotting β -galactosidase specific activity (mUnits/mg protein) vs. increasing concentration of zinc (μ M) inducer for cell line 293 (MT-ORF6) transduced with AVCMV~~LacZ~~ (first row below each bar). Also provided is the fold-induction relative to 293 cells (second row below each bar), and the fold-induction relative to 293(ORF6) cells maintained in the absence of zinc (third row).

Fig. 8B is a bar graph plotting CPM of duplex monomer replicative form (RFm) of rAAV vs. the concentration of zinc (μ M) used for induction and the fold-induction relative to 293(ORF6) cells maintained in 0 mM zinc below each bar.

Fig. 8C is a graphical comparison of the induction profiles that describe AV.CMV LacZ transduction efficiency. Specific activity data from Fig. 8A and CPM data of AV.CMV LacZ RfM from Fig. 8B are plotted along the vertical axis, and concentration of zinc sulfate used during the experiment is shown along the horizontal axis.

Fig. 9 is a bar graph plotting specific activity (milliunits β -galactosidase/mg protein) vs the concentration of zinc used for induction (first row under the horizontal axis), the fold-induction relative to HeLa cells (second row), and the fold-induction relative to HeLa(MT-ORF6) cells maintained in the absence of zinc (third row), for the HeLa(MT-ORF6) cells transduced at an MOI of 1,000 AV.CMV LacZ virus particles/cell in the absence of zinc sulfate inducer or in the presence of 50, 100, 150, 200 or 250 μM zinc sulfate inducer.

Fig. 10 is a schematic of the plasmid pAV.CMV LacZ [SEQ ID NO: 4].

Fig. 11 illustrates plasmid pAV.CMV ALP .GRE-ORF6 [SEQ ID NO: 5].

Detailed Description of the Invention

The present invention provides packaging cell lines, which enable the production of recombinant adenoviruses (rAd) functionally deleted in both the E1 and E4 genes. These rAd and methods which enable the therapeutic treatment of disorders with such rAds are disclosed. Novel "second generation" recombinant adeno-associated virus (rAAV) and methods for enhancing the transduction efficiency of rAAV containing a transgene for expression in a somatic gene therapy protocol are also provided. The methods and compositions of this invention are useful in ex vivo applications of gene therapy, such as in the transduction of bone marrow cells with desirable hematopoietic stem cell progenitor genes prior to bone marrow transplantation. The embodiments of the invention

are also useful in pharmaceutical compositions for direct *in vivo* treatment of patients by gene therapy vectors, including the transduction of desirable genes in patients with genetic disorders, such as cystic fibrosis.

5 I. Packaging Cell Lines

To increase the transgene capacity and decrease
immune response of rAds, as many viral genes as possible
should be deleted to inactivate the adenovirus. However,
it is crucial to generate complementing cell lines for
10 construction and propagation of such deleted Ad. The
method and compositions of the present invention overcome
several problems previously identified in the gene
therapy for first generation E1 deleted adenoviruses and
display advantages in administration particularly to
15 muscle tissue.

Early region 4 (E4) of Ad serotype 5 consists of 7
ORFs believed to be involved in viral DNA replication,
host cell shut-off, and late mRNA accumulation. To
generate rAd deleted in E4, the function of the E4 region
20 must be supplied to the rAd by a helper virus or
packaging cell line. However, useful packaging cell
lines have not been available previously because normally
the continuous expression of functioning Ad E1 and
functional E4 in a single cell line are toxic to the
25 cell. Such cells are therefore not useful for the growth
and replication of rAds. Further, the DNA encoding the
functional Ad E1 and Ad E4 genes, when present in a
packaging cell line, can increase the chances of
recombination with a rAd virus to cause the virus to
30 revert to a wildtype Ad virus.

The present invention avoids these problems by
providing a packaging cell line which contains the Ad5 E1
gene and only the ORF 6 of the Ad5 E4 gene. ORF6 of E4
alone can provide the requirements for E4 in the viral
35 life cycle.

According to this invention, the ORF6 is preferably under the transcriptional control of an inducible promoter. The mouse mammary tumor virus (MMTV) promoter, inducible by a glucocorticoid, particularly, dexamethasone, is presently preferred. The DNA sequence of the MMTV promoter spans nucleotides 1-1506 of SEQ ID NO: 1. Another inducible promoter is the sheep metallothionine (MT) promoter, inducible by zinc [M. G. Peterson et al, Eur. J. Biochem., 174:417-424 (1988)]. However, the zinc sulfate inducer of the MT promoter can itself be toxic to the cells. Other inducible promoters, such as those identified in International patent application WO95/13392, published May 18, 1995, and incorporated by reference herein may also be used in the production of packaging cell lines according to this invention. Constitutive promoters, such as the constitutive Ad5 E4 region promoter, LTR, may be employed in control of the expression of ORF6.

The packaging cell line of the invention which utilizes an inducible promoter permits one to control the development of toxicity by regulating the expression of the E4 ORF6 gene. After the desired shuttle vector containing the Ad sequences is transfected into the cell line, expression of the E4 ORF6 can be induced by the appropriate inducer. The packaging cell is thus able to provide both Ad E1 and Ad E4 ORF6 gene products to the rAd for a sufficient period to allow productive infection and recovery of the rAd, before the cell becomes toxic. At present, the time period before the cell experiences toxicity is about 10 days.

In its most preferred form, the packaging cell line is a human embryonic kidney (HEK) 293 E1 expressing cell line into which is introduced the E4 ORF 6 sequence under the control of the inducible promoter. It should be understood by one of skill in the art that another parent

cell line may be selected for the generation of a novel cell line expressing the E1a, E1b, and E4 ORF6 genes of a selected adenovirus serotype. Among such parent cell lines may be included HeLa [CCL 2], A549 [CCL 185], KB
5 [CCL 17], Detroit [e.g., Detroit 510, CCL 72] and WI-38 [ATCC CCL 75] cells. These cell lines are all available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA. Other suitable parent cell lines may be obtained from other sources. If such parent
10 cell lines were selected for modification, the cell line would need to be further supplied with the E1a and E1b gene functions, e.g., such as by transfection with a plasmid containing these genes or functional fragments thereof under a suitable promoter, as well as with the
15 ORF6 gene as described herein.

Example 1 teaches construction of packaging cell lines containing only the ORF 6 of Ad5 E4 region or, for functional comparisons, the entire E4 region. Briefly described, the entire E4 region and an ORF6 sequence of
20 Ad 5 E4 gene were obtained by known techniques [see, e.g., Sambrook et al., "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989) and references cited therein]. To isolate the ORF6 region, the anchored PCR technique was used to
25 amplify the ORF6 sequence from its initiation codon to its termination codon. Primers selected from the published sequence of ORF6 were used to amplify the ORF sequence and insert restriction sites onto the end of the sequence. The E4 ORF6 sequence itself is reproduced as
30 nucleotides 1523 through 2408 of SEQ ID NO: 1. The entire E4 gene sequence is published in the Genbank sequence of Ad5 [Genbank Accession No. M73260].

A minigene was constructed that placed the ORF6 sequence under the control of a selected promoter. By
35 "minigene" as used here is meant the combination of the

desired sequence to be expressed (in this particular instance, the ORF6 sequence) and the other regulatory elements necessary to transcribe the desired sequence and express the gene product in a cell containing that minigene. The ORF6 sequence gene is operatively linked to regulatory components in a manner which permits its transcription. Such components include conventional regulatory elements, such as a promoter to drive ORF6 expression. One inducible promoter was the Zn^{+2} inducible MT promoter; the other was the dexamethasone-inducible MMTV promoter of SEQ ID NO: 1.

The minigene also contains nucleic acid sequences heterologous to the ORF6 viral sequence, including sequences providing signals required for efficient polyadenylation of the transcript (poly-A or pA). A common poly-A sequence which is employed in this invention is that derived from the growth hormone (GH) gene terminator sequence (nuc. 2409-3654 of SEQ ID NO: 1). The poly-A sequence generally is inserted in the minigene following the ORF6 sequence. The polyA sequence employed in the MMTV-ORF6 minigene described in Example 1 [SEQ ID NO: 1] is supplied by the GH gene terminator and an SV40 origin of replication (ori). A similar minigene differing in promoter sequence, polyA sequence and/or SV40 ori can also be designed by one of skill in the art to transfer the E4 ORF6 sequence to a shuttle plasmid. Selection of these and other common vector elements are conventional [see, e.g., Sambrook et al, cited above, and references cited therein] and many such sequences are available from commercial and industrial sources as well as from Genbank.

The ORF6-containing minigene was subcloned into a pBR322-based shuttle plasmid that contained a neomycin resistance gene, resulting in the shuttle vector of Fig. 1. Any of the many known bacterial shuttle vectors may

be employed to carry the minigene, providing that the vector contains a reporter gene or selectable marker of which many, e.g., neo, amp or purimycin, are known in the art. It is expected that one of skill in the art can
5 develop other suitable shuttle vectors using other plasmid components which are similarly capable of transferring the ORF6 minigene into the chromosome of a
cell transfected with the plasmid.

As further described in Example 1, other shuttle
10 vectors were designed for comparative purposes, which contain the complete or substantially complete Ad5 E4 region under the control of the constitutive retroviral MLV LTR sequence in the presence or absence of the endogenous E4 promoter. The shuttle plasmid carrying the
15 ORF6 minigene (or the entire E4 region) was introduced into HEK 293 cells which express the Ad E1 gene products. Complementing cell lines were generated that express these Ad E4 or ORF6 genes from either their endogenous promoters or heterologous inducible promoters. These
20 cell lines are further characterized by their genetic constitution, E4 protein synthesis, recombinant AAV helper function, relative plaque efficiency of H5dl1004 virus, and growth kinetics of recombinant E1/E4 deleted adenovirus. These characteristics of exemplary E1/E4
25 expressing packaging cell lines are discussed in detail in the following examples.

II. Recombinant Adenovirus

The E1/E4 expressing cell line is useful in constructing E1/E4 deleted rAds which can deliver a
30 suitable gene to mammalian cells and tissues. These rAd are functionally deleted in at least the E1a, E1b and E4 Ad gene regions. By the term "functionally deleted" is meant that a sufficient amount of the gene region is removed or otherwise damaged, e.g., by mutation or
35 modification, so that the gene region is no longer

capable of producing the products of gene expression. If desired, the entire gene region may be removed. In *in vivo* experiments with the rAd grown in the packaging cell lines, the E1/E4 deleted rAd demonstrated utility particularly in transferring a transgene to a muscle cell.

The adenovirus sequences used in the construction of the shuttle vectors, helper viruses, if needed, and rAd, and other components and sequences employed in the construction of the vectors and viruses described herein may be readily obtained from commercial or academic sources based on previously published and described sequences. Viral materials may also be obtained from an individual patient. The viral sequences and vector components may be generated by resort to the teachings and references contained herein, coupled with standard recombinant molecular cloning techniques known and practiced by those skilled in the art. Modifications of existing nucleic acid sequences forming the vectors, including sequence deletions, insertions, and other mutations taught by this specification may be generated using standard techniques. Similarly, the methods employed for the selection of viral sequences useful in a vector, the cloning and construction of the "minigene" and its insertion into a desired viral shuttle vector and the production of a recombinant infectious virus are within the skill in the art given the teachings provided herein.

A. Construction of the Transgene

A "minigene" in this context is defined as above, except that the components of this minigene are designed to express the gene product *ex vivo* or *in vivo*. Such components include conventional regulatory elements necessary to drive expression of the transgene in a cell transfected with the rAd. For this minigene, a selected

promoter is operatively linked to the transgene and located, with other regulatory elements, within the selected viral sequences of the recombinant vector. Selection of the promoter is a routine matter and is not
5 a limitation of this invention. Useful promoters may be constitutive promoters or regulated (inducible)

promoters, which will enable control of the amount of the transgene to be expressed. For example, a desirable promoter is that of the cytomegalovirus (CMV) immediate
10 early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. Another desirable promoter includes the Rous sarcoma virus LTR promoter/enhancer. Still another promoter/enhancer sequence is the chicken cytoplasmic β -actin (CB) promoter [T. A. Kost et al,
15 Nucl. Acids Res., 11(23):8287 (1983)]. Other suitable promoters may be selected by one of skill in the art.

The minigene may also desirably contain nucleic acid sequences heterologous to the viral vector sequences including poly-A sequences and introns with functional
20 splice donor and acceptor sites, as described above. The poly-A sequence generally is inserted in the minigene following the transgene sequences and before the 3' adenovirus sequences. A minigene of the present invention may also contain an intron, desirably located
25 between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional as described above and many such sequences are available from commercial and industrial sources as well as from Genbank.

30 As above stated, the minigene is located in the site of any selected deletion in the rAd. In the E1/E4 deleted rAd H5.001CBLacZ, the transgene is located in the deleted E1 gene region. However, the transgene may be located elsewhere in the adenovirus sequence, as desired.

B. Production of Recombinant Adenovirus

Adenovirus sequences useful in this invention may include the DNA sequences of a number of adenovirus types, which are available from Genbank, including type
5 Ad5 [Genbank Accession No. M73260]. The adenovirus sequences may be obtained from any known adenovirus serotype, such as serotypes 2, 3, 4, 7, 12 and 40, and
further including any of the presently identified 41
10 human types [see, e.g., Horwitz, cited above]. Similarly adenoviruses known to infect other animals may also be employed in the vector constructs of this invention. The selection of the adenovirus type is not anticipated to limit the following invention. A variety of adenovirus strains are available from the American Type Culture
15 Collection, Rockville, Maryland, or available by request from a variety of commercial and institutional sources. In the following exemplary embodiment an adenovirus, type 5 (Ad5) is used for convenience.

However, it is desirable to obtain a variety of
20 adenovirus shuttle vectors based on different human adenovirus serotypes. It is anticipated that a library of such plasmids and the resulting rAds would be useful in a therapeutic regimen to evade cellular, and possibly humoral, immunity, and lengthen the duration of transgene
25 expression, as well as improve the success of repeat therapeutic treatments. Additionally the use of various serotypes is believed to produce rAd with different tissue targeting specificities. Additionally, the absence of adenoviral genes E1 and E4 in the rAd of this
30 invention should reduce or eliminate adverse CTL responses which normally cause destruction of rAds deleted of only the E1 gene.

rAds of this invention are recombinant, defective adenoviruses (i.e., E1 deleted) which are also
35 deleted completely or functionally of the E4 gene region.

Functional deletions of E4 gene regions may be assessed by assays of Examples 2 and 3, among other assays. rAds useful in this invention may optionally bear other mutations, e.g., temperature sensitive (ts) mutations in the E2a gene region, and deletions in the E3 gene regions.

An adenovirus of this invention contains a functional deletion of the adenoviral early immediate early gene E1a (which spans mu 1.3 to 4.5) and delayed early gene E1b (which spans mu 4.6 to 11.2). Similarly the adenovirus has a functional deletion of the whole E4 region (which spans mu 92 to 97.2), or of at least ORF6 of the E4 region. Gene regions which may be optionally deleted in the E1/E4 deleted rAd of this invention include all or a portion of the adenovirus delayed early gene E3 (which spans mu 76.6 to 86.2). The function of E3 is irrelevant to the function and production of the rAd.

The rAd of this invention may also have a mutation which results in reduced expression of adenoviral protein and/or reduced viral replication. For example, a ts mutation may be introduced into the adenovirus delayed early gene E2a (which spans mu 67.9 to 61.5). Among such mutations include the incorporation of the missense ts mutation in the (DBP)E2a region found in the Ad5 H5ts125 strain [P. Vander Vliet et al, J. Virol., 15:348-354 (1975)] at 62.5 mu. A single amino acid substitution (62.5 mu) at the carboxy end of the 72 kd protein produced from the E2a gene in this strain produces a protein product which is a ss DNA binding protein and is involved in the replication of adenoviral genomic DNA. At permissive temperatures (approximately 32°C) the ts strain is capable of full life cycle growth on HeLa cells, while at non-permissive temperatures (approximately 38°C) no replication of adenoviral DNA is

seen. In addition, at non-permissive temperatures, decreased immunoreactive 72 kd protein is seen in HeLa cells. See, e.g., J. F. Engelhardt et al, Hum. Gene Ther., 5:1217-1229 (1994); J. F. Engelhardt et al, Proc. Natl. Acad. Sci., USA, 91:6196-6200 (1994) and International patent application WO95/13392, published May 18, 1995, incorporated by reference herein.

However, it should be understood that other deletions in the adenovirus genome as previously described in the art or otherwise may also occur in the rAds of this invention. One minimal type of rAd can contain adenovirus genomic sequences from which all viral genes are deleted. More specifically, the adenovirus sequences may be only the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as oris) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. The adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region (Ad5 mu 0-1 or bp 1-360) can be employed as the 5' adenovirus sequence in rAd of this invention. The 3' adenovirus sequences including the right terminal (3') ITR sequence of the adenoviral genome spanning about bp 35,353 - end of the adenovirus genome, or map units 98.4-100 may be desirably employed as the 3' sequence of the rAd. These sequences, which are clearly devoid of the E1 and E4 genes, can flank, or be operatively associated with the minigene in a rAd. Any other necessary Ad gene products will then be supplied by helper viruses and the E1/E4 ORF6 expressing packaging cell of this invention.

Exemplary rAd for use in this invention, for example, may be obtained by homologous recombination of desired fragments from various rAd, a technique which has been commonly employed to generate other rAd for gene

therapy use. In the examples below, a representative rAd, H5.001CBLacZ, is constructed by homologous recombination between the adenovirus dl1004 (also H5dl1004) viral backbone and pAdCBLacZ minigene DNA.

5 H5dl1004 is an Ad5 virus deleted of from about map unit 92.1 through map unit 98, i.e, substantially the entire E4 gene. The dl1004 virus is described in Bridge and Ketner, J. Virol., 632(2):631-638 (Feb. 1989).

The pAdCBLacZ vector is a cDNA plasmid
10 containing Ad m.u. 0-1, an E1 deletion into which is inserted a bacterial β -galactosidase gene under the control of a chicken β -actin promoter, with other regulatory elements as described below, and flanked by Ad m.u. 9-16 and plasmid sequence.

15 The production of the E1/E4 rAd of this invention in the packaging cell line of this invention utilizes conventional techniques. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, cited above],
20 use of overlapping oligonucleotide sequences of the adenovirus genomes, PCR and any suitable method which provides the desired nucleotide sequence. Standard transfection and co-transfection techniques are employed, e.g., CaPO₄ transfection techniques using the
25 complementation 293 cell line. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

For example, following the construction and
30 assembly of the desired minigene-containing plasmid vector pAdCBLacZ, the E1/E4 expressing packaging cell line of this invention is infected with the helper virus H5dl1004. The infected cell line is then subsequently transfected with an adenovirus plasmid vector by
35 conventional methods. Homologous recombination occurs

between the E4-deleted H5dl1004 helper and the pAdCBLacZ vector, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the rAd. About 30 or more hours post-transfection, the cells are harvested, an extract prepared and the rAd containing the LacZ transgene is purified by buoyant density ultracentrifugation in a CsCl gradient.

III. Use of the Recombinant Virus in Gene Therapy

The rAd containing the transgene produced by cooperation of the adenovirus vector and E4 deleted helper virus and packaging cell line, as described above, provides an efficient gene transfer vehicle which can deliver the transgene in a pharmaceutical composition to a patient *in vivo* or *ex vivo* and provide for integration of the gene into a mammalian cell.

The rAd are administered to humans in a conventional manner for gene therapy and serve as an alternative or supplemental gene therapy for the disorder to which the transgene is directed. A rAd of this invention may be administered to a patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

The rAd are administered in sufficient amounts to transfect the desired target cells, e.g., muscle, liver, epithelial, etc. and provide sufficient levels of transfer and expression of the transgene to provide a therapeutic benefit without undue adverse or with medically acceptable physiological effects which can be determined by those skilled in the medical arts.

Conventional and pharmaceutically acceptable routes of administration include direct delivery to the muscle or other selected cell, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other parental routes of administration. Routes of administration may be combined, if desired.

Dosages of rAd will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective human dose of the rAd is generally in the range of from about 20 to about 100 ml of saline solution containing concentrations of from about 1×10^9 to 1×10^{11} pfu/ml virus. A preferred human dose is estimated to be about 50 ml saline solution at 2×10^{10} pfu/ml. The dose will be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the transgene can be monitored to determine the frequency of administration.

An optional method step involves the co-administration to the patient, either concurrently with, or before or after administration of the rAd of a suitable amount of a short acting immune modulator. The selected immune modulator is defined herein as an agent capable of inhibiting the formation of neutralizing antibodies directed against the recombinant vector of this invention or capable of inhibiting or substantially delaying cytolytic T lymphocyte (CTL) elimination of the vector. Among desirable immune modulators are interleukin-12 [European Patent Application No. 441,900]; gamma interferon [S. C. Morris et al, J. Immunol., 152:1047 (1994)]; interleukin-4 [United States Patent No. 5,017,691]; antibody to the CD4 protein, such as anti-OKT 3+ [see, e.g., US Patent No. 4,658,019] or antibody GK1.5 (ATCC Accession No. TIB207); a soluble CD40 molecule or an antibody to CD40 ligand (Bristol-Myers Squibb Co)

[European patent application 555,880, published August 18, 1993]; a soluble form of B7 or an antibody to CD28 or CTLA4 [CTLA4-Ig (Bristol-Myers Squibb Co), European patent application 606,217, published July 20, 1994], or
5 agents such as cyclosporin A or cyclophosphamide. Thus, the pharmaceutical compositions and methods of this invention provide a desirable gene therapy treatment.

IV. Recombinant Adeno-Associated Virus

In the following context the term "transgene" means
10 a nucleic acid sequence or reverse transcript thereof, heterologous to the AAV sequence, which encodes a polypeptide or protein of interest. The transgene may be operatively linked to regulatory components in a manner which permits transgene transcription, i.e., the
15 transgene is placed into operative association with a promoter, as well as other regulatory sequences, such as SV40 introns or polyA sequences, useful for its regulation. The composite association of the transgene with its regulatory sequences is referred to herein as a
20 minicassette or minigene.

The composition of the transgene or minicassette sequence will depend upon the use to which the resulting rAAV will be put. For example, one type of transgene sequence includes a reporter sequence, which upon
25 expression produces a detectable signal. Such reporter sequences include without limitation, an *E. coli* β -galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene (ALP) and a green fluorescent protein gene. These sequences, when associated with regulatory elements which
30 drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc.

Another type of transgene sequence includes a therapeutic gene which expresses a desired gene product
35 in a host cell. These therapeutic nucleic acid sequences

typically encode products for administration and expression in a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease. Such transgenes
5 may be readily selected by one of skill in this art and the design of the transgene or the minicassette for insertion into the rAAV is not a limitation of this invention.

The term "rAAV" encompasses any recombinant AAV gene
10 therapy vehicle of the prior art, including the AdAAV hybrid virus described in published International Patent Application No. WO96/13598, published May 9, 1996. More specifically, rAAV defines a rAAV comprising: (a) the DNA of at least a portion of the genome of an AAV, which
15 portion is capable of transducing into a target cell at least one selected gene in the absence of cell division; and (b) at least one selected gene (or transgene) operatively linked to regulatory sequences directing its expression, the gene flanked by the DNA of (a) and
20 capable of expression in the target cell *in vivo* or *in vitro*.

Other rAAVs have been described in the art. The method of this invention is not limited by the precise nature of the AAV sequences used in the rAAV, provided
25 that at a minimum both the 5' and 3' AAV inverted terminal repeats are present. Thus, the rAAV may be selected by one of skill in the art, and is not itself a limitation on this invention. The rAAVs specifically disclosed herein are illustrative.

By the term "transduction" is meant that the rAAV
30 produced by practice of the invention is capable of infecting a desired target cell and expressing the transgene in the cell by harnessing the cell's machinery. Transduction may include stably integrating the viral DNA
35 into a chromosome of the target cell. "Enhanced

transduction" is defined as the ability of the rAAV in the presence of a conversion agent to transduce the target cell, either *in vitro*, *ex vivo* or *in vivo*, at an efficiency greater than a typical prior art rAAV produced in, and purified from, a culture co-infected with an adenovirus or herpesvirus helper.

This method is based on the observation that the limiting step in rAAV mediated transduction of cells for gene therapy is not the internalization or transfer of the ss viral genome, but rather the subsequent conversion of the single-stranded (ss) viral genome to a transcriptionally active double-stranded (ds) form. Formation of ds DNA intermediates is necessary for recombinant gene expression, which is likely to be modulated by viral and cellular factors through posttranscriptional mechanisms. The inventors have designed a method to overcome this rate-limiting step, thereby enhancing transduction ability of an rAAV and ultimately the use of rAAV in gene therapy protocols.

This method of the present invention may employ a conventionally prepared ss rAAV containing a transgene. The prior art produces ss rAAV by co-infection in culture with a helper adenovirus or herpesvirus, followed by purifying the rAAV from the culture contaminants including the helper virus, and infecting the target cell with the rAAV alone. The present invention provides for infecting a target cell with a ss rAAV. However, once the target cell is infected, the infected cell is contacted with an agent which facilitates the conversion of the ss rAAV to the ds form of rAAV. The action of this "facilitating agent" or "conversion agent" causes the ss to ds conversion to occur in the target cell, resulting in enhanced transduction of the recombinant AAV into the target cell. By facilitating the conversion of ss to ds rAAV in the target cell, the method of this

invention may also result in both transduction and stable chromosomal integration of the rAAV into the chromosome of said host cell.

Preferably, for use of this invention the
5 "facilitating or conversion agent" may take several forms.

~~A. The Conversion Agent is a Helper Virus~~

In one embodiment, the agent is a helper virus and the method includes an additional step of co-
10 infecting the target cell with the helper virus. The helper virus useful in this method contains a selected gene which can facilitate the conversion of ss rAAV to ds rAAV. The selected gene may encode a gene product or polypeptide (or a functional fragment of the polypeptide
15 which shares the biological activity of the full-length polypeptide) which enhances the conversion. Alternatively, the selected gene may express an antisense or ribozyme which functions in the cell to block or inhibit a cellular gene that normally prevents ss to ds
20 conversion of the rAAV. These genes may also be employed in the second generation rAAV described below.

The helper virus is capable of expressing the selected gene product in the target cell in the absence of cell division. The helper virus may be a wild-type or
25 mutant adenovirus. The helper virus may alternatively be a wild-type or mutant herpesvirus. Preferably, for use as facilitating agents, such viruses are mutants deleted of several normal genes so that the helper viruses and/or their expressed gene products will not cause disease in a
30 patient.

For example, a helper adenovirus useful in this invention may express only a gene product of a single adenoviral early gene. Exposure of the ss rAAV to an Ad
early gene product is sufficient to substantially enhance
35 the formation of ds rAAV genome with a coordinate

increase in transduction efficiency. The Ad early genes which are useful in producing this effect are E1, E2a, E4 and functional fragments thereof. However, as demonstrated by the examples below, adenovirus

5 substantially enhances recombinant AAV transduction in vitro in a way that is dependent on expression of the E1 and E4 genes of adenovirus and is directly proportional to the appearance of ds replicative forms of rAAV.

One example of a helper virus is an adenovirus

10 deleted of most of its wild-type early genes and which is capable of expressing only its E4 gene or a functional fragment thereof in the target cell. Among such functional fragments is the ORF 6 of the E4 gene. As described below in the examples, experiments in cell

15 lines indicate that the ORF6 of the adenoviral E4 gene locus is sufficient to significantly enhance rAAV transduction. Selective expression of the E4-ORF6 product of adenovirus accomplishes a increase in transduction efficiency similar to, but somewhat

20 attenuated, compared to that produced by exposure to the E1 and E4 gene products in combination. That is, the ORF6 product of E4 is sufficient to enhance the augmentation of rAAV transduction; but this effect is amplified substantially by E1 gene products.

25 Thus, more preferably, exposure of the rAAV to both the expressed E1 and E4 gene products produces a substantial enhancement of the above-described rate limiting step. Therefore, another exemplary helper virus may also contain more than one gene which, upon

30 expression, facilitates the ss to ds conversion. An example of such a helper virus is an adenovirus which expresses both the E1 and E4 genes, or functional fragments thereof. Still other Ad genes may be expressed by the helper virus, provided that the virus is

35 sufficiently crippled so that it does not cause disease

in the patient contributing the target cells.

Where the agent which facilitates conversion of ss to ds rAAV is a helper virus, the method of the invention comprises co-infecting the target cell with the rAAV and the helper virus. Such co-infection may occur in the context of ex vivo therapy, i.e., manipulations performed on cells extracted from the patient, which cells are reinserted into the patient after the method is performed. Alternatively, the patient may be directly co-infected with the two viruses by conventional means. Delivery of the two viruses to the patient may be directed to a specific organ, or to the general circulatory system. Such delivery methods are described in the art for gene therapy of, e.g., cystic fibrosis [see, e.g., US Patent No. 5,240,846].

B. The Conversion Agent is a Chemical, Drug or Other Entity that can Activate rAAV Transduction

In another embodiment of the method of this invention, the conversion agent which contacts the cells infected with the rAAV may be selected from the following classes of known compounds or methods: 1) inhibitors of DNA synthesis such as hydroxyurea, hydrogen peroxide, and other direct or indirect inhibitors of DNA polymerase; 2) chemo-therapeutic agents that induce DNA damage, such as cyclophosphamide, alkylating agents, purine analogs, e.g., 6-thioguanine, etc.; 3) drugs that interfere with DNA modifying enzymes, such as inhibitors of topoisomerase, DNA ligase exonucleases and endonucleases; and 4) agents that nonspecifically enhance transcription, such as sodium butyrate, or agents that stabilize cells, such as DMSO. Also, genotoxic agents such as carcinogens may be employed as the conversion agent. Other methods of inducing disruption or damage to DNA may also be useful as agents capable of facilitating ss to ds conversion of rAAV and maybe selected by one of skill in

the art, including physical methods, such as irradiation. These classes of compounds or methods are believed to result in the conversion from ss to ds rAAV.

According to this embodiment of the method of
5 the invention, the rAAV is again produced conventionally, but not co-infected with a helper virus. The ss rAAV is
~~infected into the target cell, and the infected cell is~~
contacted by the agent in an appropriate manner depending on the identity of the agent. These conversion enhancing
10 agents can be employed in ex vivo treatment of the target cells infected by the rAAV by application directly to the cells. Such application can occur substantially simultaneously, or consecutively, with application of the rAAV gene therapy vehicle. For example, the infected
15 target cell may be subjected to one of the above-listed compounds or drugs for a desired time period. The parameters for contacting the infected cells with the agent may readily be determined by one of skill in the art. These parameters will depend upon whether the
20 method is performed ex vivo or in vivo. For example, the number of ex vivo infected cells to be treated will be considered for the dosage, and timing of such treatment.

Similarly, the physical status of the patient can determine the parameters of delivery of the agent to
25 the patient in vivo. The dosage and amount of the damaging agent may therefore be adjusted by one of skill in the art. Where the agents are typical chemotherapeutic drugs approved for use in humans or animals, such enhanced conversion of rAAV may also occur
30 in vivo by the co-administration of the agent, i.e., the chemotherapeutic drug, and the rAAV gene therapy vehicle to the patient. According to this aspect of the invention, the chemotherapeutic drug would be administered only when the rAAV is administered.
35 Appropriate dosages and amounts of chemotherapeutic drugs

and recombinant gene therapy vehicles and means for determining such amounts are within the skill of the art. However, because the effect of the chemotherapeutic drug will enhance the ss to ds conversion of the rAAV and thus
5 enhance its efficiency of transduction into the target cells, it is anticipated that lower dosages than the conventional dosages of either or both the drug and the rAAV could be effectively administered.

C. Conversion Agent May be Part of the rAAV.

10 In still another embodiment of this invention, a novel "second generation" rAAV may be designed to incorporate the conversion agent into the virus, so that both the transgene and the conversion agent are co-expressed in the target cell. Such a novel recombinant
15 adeno-associated virus comprises the following components:

(a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing at least two selected genes or
20 functional fragments thereof into a target cell in the absence of cell division; (b) a first selected gene, i.e., the desired transgene, operatively linked to regulatory sequences directing its expression, and (c) a second selected gene, i.e., the "conversion gene"
25 operatively linked to regulatory sequences capable of directing expression of said second gene. The "conversion gene" upon expression is capable of facilitating the conversion of the ss rAAV to its ds form upon expression. The first and second genes in this rAAV
30 are flanked by the AAV DNA, preferably the 5' and 3' ITRs. An embodiment of such a second generation rAAV is provided schematically in Fig. 11. Its DNA sequence is provided in SEQ ID NO: 5.

Another embodiment of such a novel rAAV may
35 include more than one gene which upon expression has the

ability to facilitate conversion of ss to ds rAAV in the target cell. For example, the novel rAAV described above may also contain an additional selected gene operatively linked to regulatory sequences capable of directing its expression, the additional gene and said second "conversion" gene described above being capable of jointly facilitating the conversion of ss rAAV to its ds form upon expression of both the second and additional genes. In this rAAV, all three genes, i.e., the transgene, the second "conversion" gene and the additional gene are flanked by the AAV DNA.

In one desirable embodiment of a novel rAAV the AAV ITRs flank a selected transgene, and a conversion gene, which is the adenovirus E4 gene or a functional fragment thereof (e.g., the ORF6 sequence). In another embodiment, the novel recombinant expresses three genes, the transgene, the adenovirus E4 gene or a functional fragment thereof and the adenovirus E1 gene or a functional fragment thereof. The E4 and E1 gene products expressed in the target cell with the transgene, together act to facilitate conversion of the ss to ds form of rAAV.

In still another embodiment of the novel rAAV and its use, the regulatory sequences directing expression of the conversion gene, e.g., whether it be a single second gene or more than a single additional gene, may include an inducible promoter. Thus, expression of the conversion gene occurs only in the presence of an inducing agent. Many inducible promoters and companion inducing agents, e.g., steroids such as glucocorticoids, are known to the art and may be readily selected for incorporation into the rAAV and methods of this invention by one of skill in the art with resort to this description.

The method of the invention employing such

"second generation" rAAVs which carry at least one "conversion gene" provides for infecting the target cell with this ss rAAV. Where the promoters directing expression of both the transgene and the conversion gene are constitutive, the infected target cell machinery will direct the expression of the transgene product and conversion gene product. Co-expression in the target cell of the transgene and the "conversion gene" facilitates the conversion of ss rAAV to ds rAAV in the cell, and increases the transduction efficiency, and perhaps stable chromosomal integration, without further method steps.

When the second generation rAAV employed in the method contains the "conversion gene(s)" under the control of inducible promoter(s), the method is slightly altered. Following infection of the target cell by the rAAV, the infected target cell is contacted with a suitable inducing agent, which triggers the inducible promoter to "turn on" production of the conversion gene product. When the inducing agent is removed or stopped, the expression of the conversion gene product is "turned off".

As described above, any prior art rAAV containing a transgene for gene therapy may be used in at least one embodiment of the above methods. The sources, selection and assembly of the various components to generate the rAAV, including the novel rAAV described above, are now conventional and readily accessible to one of skill in this art, given the disclosure contained herein. Such methods employ conventional genetic engineering techniques [See, e.g. Sambrook et al, cited above].

The novel rAAV viruses and the methods of this invention provide efficient gene transfer vehicles for somatic gene therapy and are suitable in pharmaceutical

compositions for *ex vivo* applications and *in vivo* use. When rAAV contain a therapeutic gene, e.g., in place of the *LacZ* transgene illustrated in the exemplary rAAV, AV.CMV*LacZ*, by use of the rAAV and the methods described
5 herein, the therapeutic transgene can be delivered to a patient *in vivo* or *ex vivo* to provide for efficient transduction, and possibly stable integration, of the desired gene into the target cell. Thus, these novel rAAV and the methods described herein can be employed to
10 correct genetic deficiencies or defects. The potential of AAV to efficiently integrate its genome into nondividing cells is currently being exploited in the development of gene therapies based on *ex vivo* transduction of hematopoietic stem cells. *In vivo*
15 application of rAAV is primarily being developed for the treatment of CF where purified stocks of virus are instilled into the airway to transduce the terminally differentiated epithelial cells of conducting airway. The methods and compositions described herein can be used
20 with both types of gene therapy. Another condition suitable for such use includes transduction of the low density lipoprotein (LDL) receptor gene into hepatocytes for the treatment of familial hypercholesterolemia. One of skill in the art can generate any number of rAAV which
25 can be used via the above methods for the treatment of these and other disorders.

For *ex vivo* or for *in vivo* therapy, the rAAV may be used to infect the target cells by suspending the virus particles in a biologically compatible solution or
30 pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to

those of skill in the art may be employed for this purpose.

The rAAV are administered in sufficient amounts to transfect the desired cells and provide sufficient levels of expression of the selected transgene to provide a therapeutic benefit without undue adverse, or with medically acceptable, physiological effects which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of *in vivo* administration include direct delivery to the target organ, tissue or site, intranasal, intravenous, intramuscular, subcutaneous, intra~~der~~mal, oral and other parental routes of administration. Routes of administration may be combined, if desired.

Dosages of the rAAV for the infecting step of the method will depend primarily on factors such as the therapeutic environment, i.e., *ex vivo* or *in vivo*; the condition being treated, the selected gene, the age, weight and health of the patient, and may thus vary among patients. A therapeutically effective dosage of the rAAV for *ex vivo* treatment will be based upon the multiplicity of infection, which is likely to range from between about 1 to about 10 transducing particles/cell. A therapeutically effective human dosage of the rAAV for *in vivo* infection according to the present invention is believed to be in the range of from about 20 to about 50 ml of saline solution containing concentrations of from about 1×10^7 to 1×10^{10} transducing viral particles/ml virus. A preferred human dosage is about 20 ml saline solution at the above concentrations. The dosage will be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the selected gene can be monitored to determine the selection, adjustment or frequency of dosage administration.

The effective amount of the facilitating agent to be administered is within the skill of the art to determine and will depend upon the identity of the agent. Known dosages of certain of the classes of chemicals and pharmaceuticals described above may be employed in this method to damage the DNA and facilitate ss to ds conversion of the rAAV. Where the agent is a gene expressed by a helper virus, the amounts of infecting virus should be similar to those amounts described above for the rAAV. Of course, where the agent is a gene present in a second generation rAAV, the identical dosages described above for the rAAV will apply.

Several embodiments of the above-described methods of this invention were confirmed in murine models of rAAV mediated gene transfer to both lung and liver. These experiments demonstrated similarly low levels of gene transfer *in vivo* by rAAV, which was increased several orders of magnitude by coinfection with E1 and E4 expressing adenovirus.

In summary, experiments were conducted to demonstrate that adenovirus enhances rAAV transduction in cultured cells. During the production and characterization of a *lacZ* recombinant AAV generated in 293 cells that were coinfecting with an E1 deleted virus, it was observed that purification of rAAV from lysates was associated with substantial loss of *lacZ* transducing activity when assayed on 293 cells. This drop in rAAV activity was particularly evident in the final step where residual contaminating helper adenovirus was removed by heat inactivation. *LacZ* transducing activity was recovered by adding adenovirus back to the purified stock of rAAV. These data provided the first indication that adenovirus could substantially enhance the transduction efficiency of rAAV.

As described in Example 10, a series of complementation groups were generated by mixing different adenovirus early gene mutants with purified LacZ rAAV, referred to as AV.CMVLacZ (see Example 2). These defined mixtures of viruses were analyzed for LacZ transduction on Hela cells (See Examples 12 and 13). An E1 deletion rAd H5.CBALP and the E4 deletion mutant dl1004 provided no significant increase in AV.CMVLacZ transduction (Fig. 5A). However, partial activity could be achieved with E1 and E4 mutants that carried less severe deletions. Both dl110 (E1B-55kDa deleted) and dl1010 (ORF6 deleted) enhanced transduction to levels that approached those of Ad5, ts125, and dl802 in terms of the number of positive blue cells, but total β -galactosidase activity was substantially lower (Fig. 5A). These results implicate early regions E1 and E4 in the augmentation of rAAV transduction.

The experiments described below also demonstrate that the novel rAAV which incorporates as its conversion gene, an Ad gene, such as E4, can increase transduction efficiency of the rAAV in the absence of a helper virus. As described in more detail below in Example 15 below, 293 cells were stably transfected with a genomic fragment of Ad5 spanning E4. This E1/E4 expressing cell line and the parent E1 expressing cell line (293) were infected with rAAV and analyzed for transduction. These experiments demonstrated the significance of the combined expression of E1 and E4(ORF6) in the adenovirus mediated augmentation of rAAV transduction.

In the presence of E1 and E4 expression, rAAV transduction was invariably accompanied by the appearance of ds RF monomers and dimers (Example 14). Importantly, the tight correlation between rAAV vector transduction and the accumulation of duplex forms could be achieved in

two different experimental settings; cells infected with E1/E4 expressing adenovirus (Figs. 8A and 8B), or complementing cell lines (Fig. 8C).

The following examples illustrate the construction and testing of the novel packaging cell lines, the E1/E4 deleted rAd of the present invention and the use thereof, improved methods and second generation recombinant AAV production for gene therapy of the present invention. These examples are illustrative only, and do not limit the scope of the present invention.

Example 1 - Novel E1a/E1b and E4 Expressing Packaging Cell Lines

A. Construction of E4 ORF 6 Expressing Plasmids

The entire E4 region from Ad5 or an ORF6 minigene were subcloned into a shuttle plasmid that contained a neomycin resistance gene. Two versions of ORF6 minigene were developed that differed in the promoter element. The first used a Zn+2 inducible sheep metallothionine (MT) promoter to drive ORF 6 expression. The second used a dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter.

An exemplary plasmid useful for the construction of a packaging cell line of this invention is pMMTVE4ORF6. The minigene contained in this plasmid is set out in SEQ ID NO: 1, and contains a mouse mammary tumor virus promoter (MMTV) (nucleotides 1-1506 of SEQ ID NO:1) in transcriptional control of a human E4 ORF 6 gene sequence (nucleotides 1523-2408 of SEQ ID NO: 1), a growth hormone terminator (GH) (nucleotides 2409-3654 of SEQ ID NO: 1), an SV40 origin of replication, plasmid sequences from plasmid pBR322, including a neomycin resistance gene, and an ampicillin resistance gene. The amino acid sequence of ORF 6 is indicated in SEQ ID NO: 2. The various functional fragments of this plasmid may be readily replaced with other conventionally used

sequences and are not critical to the design of the plasmid.

Another plasmid useful for the construction of a packaging cell line of this invention is pMTE4ORF6.

5 The DNA sequence of the minigene contained in this plasmid is similar to that of SEQ ID NO: 1, except that the promoter is a sheep metallothionine promoter (MT promoter) [M. G. Peterson et al, cited above].

A plasmid used as a control for the
10 construction of a packaging cell line of this invention is pLTR.E4(-). This plasmid contains the endogenous constitutive retroviral MLV LTR and most of the Ad E4 gene region except that the endogenous E4 promoter and a portion of E4 ORF1 are missing. The other plasmid
15 sequences remain the same as described above.

Still another plasmid useful for the study of the methods of this invention is pLTR.E4, which contains the constitutive MLV LTR and endogenous E4 promoter and an intact E4 gene. The other plasmid sequences remain
20 the same as described above.

To determine whether ORF6 expression was sufficient to enhance rAAV transduction, the inducible metallothionein (MT)-ORF6 minigene was stably transfected into HeLa cells. This new cell line, HeLa(MT-ORF6) was
25 evaluated for LacZ rAAV transduction in response to ORF6 induction as described below. The cell line 293 (MT-ORF6) expresses ORF-6 of the E4 gene of Ad5 from the metallothionine promoter which is relatively inactive at baseline but can be induced with divalent cations. These
30 293 cells were included to establish the baseline transduction efficiency.

B. Transfections and Selection of Clones

Each of the above-described plasmids was transfected by the calcium phosphate precipitation
35 technique into the human embryonic kidney cell line 293

[ATCC CRL1573] which expresses the product of the adenovirus E1 genes, or into HeLa cells, seeded on 100 mm plates (10 µg plasmid/plate). Twenty four hours post-transfection, cells were harvested and seeded at varying dilutions (1:10 - 1:100) in 100 mm plates for about 10 days. Seeding media contain G418 (Geneticin, BRL) at 1 mg/ml. Resistant colonies that developed were selected using the following assays and expanded. Preliminary analysis of clones was based on enhanced transduction efficiency of a recombinant adeno-associated virus, AV.CMV LacZ , and immunofluorescence localization of Ad E4 protein as described in the following examples.

Example 2 - Recombinant AAV and AV.CMV LacZ Transduction Enhancement Assay

E1 and E4 Ad gene products are needed for recombinant adeno-associated virus (AAV) function. This primary assay involves seeding the packaging cell lines of Example 1 in 96 well 35 mm culture plates (2×10^6 cells/well) and infecting the cells with purified, heat-treated AV.CMV LacZ at an MOI of 1000 virus particles/cell.

A. Preparation of Recombinant AV.CMV LacZ

A recombinant AAV virus was prepared by conventional genetic engineering techniques for the purposes of this experiment. Recombinant AAV was generated by plasmid transfections in the presence of helper adenovirus [Samulski et al, *J. Virol.*, 63:3822-3828 (1989)]. A cis-acting plasmid pAV.CMV LacZ [SEQ ID NO: 4] (see Fig. 10) was derived from psub201 [Samulski et al, *J. Virol.*, 61:3096-3101 (1987)] and contains an *E. coli* β -galactosidase minigene in place of AAV Rep and Cap genes. The 5' to 3' organization of the recombinant AV.CMV LacZ genome (4.9 kb) [SEQ ID NO: 4] includes

(a) the 5' AAV ITR (bp 1-173) was obtained by PCR using pAV2 [C. A. Laughlin et al, *Gene*,

23: 65-73 (1983)] as template [nuc. 53-219];

(b) a CMV immediate early enhancer/promoter [Boshart et al, Cell, 41:521-530 (1985)] (nuc. 246-839);

5 (c) an SV40 intron (nuc. 856-987);

(d) *E. coli* β -galactosidase cDNA (nuc. 1039-4512);

(e) an SV40 polyadenylation signal (a 237 Bam HI-BclI restriction fragment containing the
10 cleavage/poly-A signals from both the early and late transcription units (nuc. 4522-4719) and

(f) 3'AAV ITR, obtained from pAV2 as a SnaBI-BglII fragment (nuc. 4759-4925). All other nucleotides are plasmid derived.

15 Rep and Cap genes were provided by a trans-acting plasmid pAAV/Ad [Samulski et al, cited above].

Monolayers of 293 cells grown to 90% confluency in 150 mm culture dishes (5×10^7 cells/plate) were infected with H5.CBALP at an MOI of 10. H5.CBALP (also
20 called H5.010ALP) is a rAd that contains an alkaline phosphatase minigene in place of adenovirus Ela and Elb gene sequences (map units 1-9.2 of the Ad5 sequence of GenBank [Accession No. M73260]). The alkaline phosphatase cDNA is under the transcriptional control of
25 a CMV-enhanced β -actin promoter in this virus. This helper virus is described in Goldman et al, Hum. Gene Ther., 6:839-851 (July, 1995); Engelhardt et al, Hum. Gene Ther., 5:1217-1229 (October, 1994); and references cited therein.

30 Infections were done in Dulbecco's Modified Eagles Media (DMEM) supplemented with 2% fetal bovine serum (FBS) at 20 ml media/150 mm plate. Two hours post-infection, 50 μ g plasmid DNA (37.5 μ g trans-acting and 12.5 μ g cis-acting) in 2.5 ml of transfection cocktail
35 was added to each plate and evenly distributed.

Transfections were calcium phosphate based as described [B. Cullen, Meth. Enzymol., 152:684-704 (1987)]. Cells were left in this condition for 10-14 hours after which the infection/transfection media was replaced with 20 ml fresh DMEM/2% FBS. Forty to fifty hours post-transfection, cells were harvested, suspended in 10 mM Tris-Cl (pH 8.0) buffer (0.5 ml/150 mm plate) and a lysate prepared by sonication. The lysate was brought to 10 mM manganese chloride, after which bovine pancreatic DNase I (20,000 units) and RNase (0.2 mg/ml final concentration) were added, and the reaction incubated at 37°C for 30 minutes. Sodium deoxycholate was added to a final concentration of 1% and incubated at 37°C for an additional 10 minutes.

The treated lysate was chilled on ice for 10 minutes and solid CsCl added to a final density of 1.3 g/ml. The lysate was brought to a final volume of 60 ml with 1.3 g/ml CsCl solution in 10 mM Tris-Cl (pH 8.0) and divided into three equal aliquots. Each 20 ml sample was layered onto a CsCl step gradient composed of two 9.0 ml tiers with densities 1.45 g/ml and 1.60 g/ml.

Centrifugation was performed at 25,000 rpm in a Beckman SW-28 rotor for 24 hours at 4°C. One ml fractions were collected from the bottom of the tube and analyzed on 293 or 293(E4) cells for *LacZ* transduction. Fractions containing peak titers of functional AV.CMV*LacZ* virus were combined and subjected to three sequential rounds of equilibrium sedimentation in CsCl. Rotor selection included a Beckman NVT-90 (80,000 rpm for 4 hours) and SW-41 (35,000 rpm for 20 hours). At equilibrium, AV.CMV*LacZ* appeared as an opalescent band at 1.40-1.41 g/ml CsCl. Densities were calculated from refractive index measurements. Purified vector was exchanged to 20 mM HEPES buffer (pH 7.8) containing 150 mM NaCl (HBS) by dialysis and stored frozen at -80°C in the

presence of 10% glycerol or as a liquid stock at -20°C in HBS/40% glycerol.

Purified virus was tested for contaminating H5.CBALP helper virus and AV.CMV LacZ titers. Helper virus was monitored by histochemical staining for reporter alkaline phosphatase activity. A sample of purified virus representing 1.0% of the final product was added to a growing monolayer of 293 cells seeded in a 60 mm plate. Forty-eight hours later, cells were fixed in 0.5% glutaraldehyde/phosphate buffered saline (PBS) for 10 minutes at room temperature, washed in PBS (3x10 minutes) and incubated at 65°C for 40 minutes to inactivate endogenous alkaline phosphatase activity. The monolayer was allowed to cool to room temperature, rinsed once briefly in 100 mM Tris-Cl (pH 9.5)/100 mM NaCl/5mM MgCl₂, and incubated at 37°C for 30 minutes in the same buffer containing 0.33 mg/ml nitroblue tetrazolium chloride (NBT) and 0.165 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP). Color development was stopped by washing the monolayer in 10 mM Tris-Cl (pH 8.0)/5 mM EDTA. Routinely the purification scheme described above removed all detectable H5.CBALP helper virus by the third round of buoyant density ultracentrifugation.

AV.CMV LacZ titers were measured according to genome copy number (virus particles/ml), absorbance at 260 nm (A_{260} particles/ml) and LacZ Forming Units (LFU/ml). Virus particle concentrations were based on Southern blotting. Briefly, a sample of purified AV.CMV LacZ was treated with capsid digestion buffer (50 mM Tris-Cl, pH 8.0/1.0 mM EDTA, pH 8.0/0.5% SDS/Proteinase K 1.0 mg/ml) at 50°C for one hour to release virus DNA. The reactions were allowed to cool to room temperature, loading dye was added and electrophoresed through a 1.2% agarose gel. Standard

quantities of ds AV.CMVLacZ genome were also resolved on the gel.

DNAs were electroblotted onto a nylon membrane, hybridized with a ^{32}P random primer labeled restriction
5 fragment, and the resulting blot scanned on a PhosphorImager 445 SI (Molecular Dynamics). A standard
curve was generated from the duplex forms and used to extrapolate the number of virus genomes in the sample.
LFU titers were generated by infecting indicator cells
10 with limiting dilutions of virus sample. Indicator cells included HeLa and 293 and 293 (E4) lines (described in Example 10 below). Twenty-four hours later, cells were fixed in glutaraldehyde and cells were histochemically stained for *E. coli* β -galactosidase (LacZ) activity as
15 described in J. M. Wilson et al, Proc. Natl. Acad. Sci. USA, 85:3014-3018 (1988). One LFU is described as the quantity of virus that is sufficient to cause visually detectable β -galactosidase expression in one cell 24 hours post-infection.

20 B. Induction of ORF6 Expression

Induction of ORF6 expression with 10 μM dexamethasone or 150 μM zinc sulfate (for negative control, no inducer used) was initiated 2 hours before the addition of virus and continued throughout the
25 duration of the experiment. Twenty-four hours after the addition of virus, cells were harvested, lysates were generated by sonication and analyzed for the β -galactosidase expression (i.e., β -galactosidase activity) and virus DNA as described above. Hirt extracts were
30 prepared from low molecular weight DNA from cell extracts. The preparation of the Hirt extracts and subsequent analysis by Southern hybridization were performed by resort to conventional procedures known to one of skill in the art.

35 In the absence of the inducers, the packaging

cell lines generate lower levels of β -galactosidase in rAAV infected cells. Induction of ORF6 expression with the inducer dexamethasone results in a concomitant rise in AV.CMVLacZ cell transduction to a level that was much greater than the parent 293 line. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction.

Results are demonstrated for certain positive clones in the Table I below (see Example 4). However, for 30 cell lines having an MMTV promoter and ORF6 sequence, 4 demonstrated over 90% blue cells illustrative of LacZ production in the presence of dexamethasone, i.e., 293-27-6, 293-27-17, 293-27-18 and 293-27-28.

Example 3 - Immunofluorescence Localization of Ad5 Late Protein.

Positive clones from the assay of Example 2 were infected with the recombinant E4 deleted adenovirus H5dl1004 and screened for E4 complementation using an immunofluorescence assay for late gene expression. The H5dl1004 virus was obtained from Dr. Ketner of Johns Hopkins University and is described in Bridge and Ketner, J. Virol., 632(2):631-638 (Feb. 1989), incorporated by reference herein. Because ORF6 of E4 complements late Ad gene expression, specifically in the formation of the hexon and penton fibers of the adenovirus, cell lines containing ORF6 are able to bind with antibody against these proteins.

Each cell line of Example 1 is infected with E4 deleted virus H5dl1004 virus at an MOI of 0.1. The cells were treated with mouse anti-adenovirus FITC-labeled monoclonal antibody to either the hexon or penton fibers in a 1:10 dilution (Chemicon International Inc., Temecula, CA). Positive clones were identified by reaction with the antibody.

Example 4 - Relative Plaquing Efficiency

The cell lines of Example 1 demonstrating with strong complementation ability in Example 3 were screened for relative plaquing efficiency of H5dl1004 as compared to W162 cells (an E4-complementing Vero cell line which does not express E1) [Weinberg and Ketner, Proc. Natl. Acad. Sci. USA, 80(17):5383-5386 (1983)]. In Table II below, RPE%, i.e., relative plaquing efficiency, represents the titer of H5dl1004 on tested cell lines/titer of H5dl1004 on W162 cells. For example, the RPE of 293 cells is 0.

The positive cell lines selected by all criteria are identified in Table I below, with the results of the assays of Examples 2, 3 and 4.

15

TABLE I

E1/E4 Double ComPLEMENTING Cell Lines

Cell Line	Trans-Gene	Pro-moter	IF/LP	AV.CMV LacZ	RPE%
293-10-3	ORF6	MT	++++	++++	246
20 293-39-11	ORF6	LTR	++++	+++	52
293-84-31	E4-	LTR	++++	++++	179
293-12-31	whole E4	LTR +E4	++++	++++	174
25 293-27-6	ORF6	MMTV		+++++	327
293-27-17	ORF6	MMTV		++++	313
293-27-18	ORF6	MMTV		+++++	339
293-27-28	ORF6	MMTV		++++	261

Example 5 - Construction and Purification of H5.001CBLacZ

30 The plasmid pAd.CBLacZ was constructed as described in detail in K. Kozarsky et al, Som. Cell Mol. Genet., 19(5): 449-458 (1993), incorporated by reference herein. This plasmid contained a minigene comprising a 5'

flanking *NheI* restriction site, followed by Ad5 sequence m.u. 0-1, followed by an *E1* deletion into which is inserted a CMV enhancer/chicken β -actin promoter sequence [T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)],
5 which controls the transcription of the following bacterial β -galactosidase, followed by a poly A sequence and flanked 3' by Ad m.u. 9-16, and another *NheI* site.

In the plasmid, the minigene was flanked on both sides by plasmid sequence containing drug resistance markers.

10 The plasmid pAd.CBLacZ was linearized with *NheI* and co-transfected by the calcium phosphate co-transfection method into the novel packaging cell line of Example 1 with *ClaI* digested H5dl1004 (an Ad5 sequence deleted of from about map unit 92.1 through map unit 98,
15 corresponding to substantially the entire *E4* gene).

Homologous recombination occurs in the cell line between these two viral constructs between Ad map units 9-16, resulting in rAd, designated H5.001CBLacZ [SEQ ID NO: 3] (Fig. 2). This rAd contains the sequence from
20 pAd.CBLacZ (including Ad map units 0-1 (nuc. 1-330); CMV enhancer/chicken β -actin promoter (CB) (nucs. 370-928); *E. coli* β -galactosidase (nucs. 945-4429); the polyA (nuc. 4429-4628); and Ad5 map units 9-92.1 and 97.3 to 100 from H5dl1004 (nucs. 4671-35408)). This rAd is thereby
25 functionally deleted, and substantially structurally deleted, of the Ad *E1* and *E4* genes.

Viral plaques were selected and screened by the β -galactosidase assay [Wilson (1988), cited above] and H5.001CBLacZ was isolated following three rounds of
30 plaque purification. The purified virus was also subjected to cesium chloride density centrifugation and large scale production.

For the following mouse experiments, virus was used after column purification and glycerol was added to a

final concentration of 10% (v/v). Virus was stored at -70°C until use.

Example 6 - Growth Kinetics of H5.001CBLacZ in Packaging Cell Lines

5 The cell lines identified in Table I were infected with recombinant H5.001CBLacZ at an MOI of .5. The growth kinetics of this virus in the E4 complementing cell lines are shown in Fig. 3. Maximum viral yield is reported as LFU/ml in Table II below.

10

TABLE II

	<u>Cell Line</u>	<u>Maximum Viral Yield</u>
	293-10-3	2.8×10^{10}
	293-39-11	9.5×10^8
	293-84-31	1.1×10^9
15	293-12-31	4.5×10^8
	293-27-6	2.8×10^{10}
	293-27-17	2.5×10^{10}
	293-27-18	2.9×10^{10}
	293-27-28	1.2×10^{10}

20 When grown in 293-27-18 cells (the E4 ORF6 cell line with MMTV promoter inducible by dexamethasone) the maximum yield of this virus is 2.9×10^{10} LFU/ml. Several of the cell lines were passaged between 5 and 20 times and the viral production of the passages remained stable.
 25 However, RPE did fall following repeated passages of cells.

Example 7 - Other Recombinant Adenoviruses

Other related rAds were prepared similarly to H5.001CBLacZ by homologous recombination between
 30 pAdCBLacZ and other helper viruses.

As one example, H5.000CBLacZ is a recombinant E1 deleted Ad5 which contains the same minigene as H5.001CBLacZ, but has an intact E4 gene. This rAd was prepared as described by homologous recombination between
 35 pAdCBLacZ and a wild-type Ad5.

As another example, H5.010CBLacZ contains the adenovirus map units 0-1, followed by a CMV enhanced, chicken cytoplasmic β -actin promoter, the *E. coli* β -galactosidase gene (lacZ), a polyadenylation signal (pA), and adenovirus type 5 map units 9-100, with a small deletion in the E3 gene (the Ad 5 sub360 backbone). This rAd may be prepared by homologous recombination between the pAdCBLacZ vector and Ad5 virus sub360, which contains a 150 bp deletion within the 14.6 kD protein of the E3 gene. See, e.g., J. F. Engelhardt et al, Proc. Natl. Acad. Sci., USA, 91:6196-6200 (June 1994); and Engelhardt et al, Hum. Gene Ther., 5:1217-1229 (Oct. 1994), both incorporated by reference herein.

These rAds were isolated following transfection [Graham, Virology, 52:456-467 (1974)], and were subjected to two rounds of plaque purification. Lysates were purified by cesium chloride density centrifugation as previously described [Engelhardt et al, Proc. Natl. Acad. Sci. USA, 88:11192-11196 (1991)]. Cesium chloride was removed by passing the virus over a BioRad DG10 column using phosphate-buffered saline.

Example 8 - LacZ Gene Transfer into Mouse

A. Transfer into Mouse Muscle

Five to six-week old male C57B/6 mice were anesthetized. Anterior tibialis muscles were exposed and directly injected with either rAd H5.000CBLacZ, H5.010CBLacZ or H5.001CBLacZ as follows: 25 μ L of purified viral suspension at a stock concentration of 5×10^{11} virus particles/mL was injected by inserting the tip of the 33 gauge needle of a 100 μ L Hamilton syringe into the belly of the muscle.

Animals were sacrificed on day 4, 14, 28 and 60 post injection. The muscles were dissected and frozen in liquid nitrogen cooled isopentane. Six μ M sections were cut in a cryostat, fixed and stained for β -galactosidase

activity for 6 hours at 37°C.

While the blue stained rAd was found for each virus in the day 4 and day 14 (most abundant) stains, by day 28, the H5.001CBLacZ clearly demonstrated more virus on day 28. By day 60, the only virus which stained positive was the H5.001CBLacZ.

B. Transfer into Mouse Lung and Circulation

Rad H5.000CBLacZ (control), and H5.001CBLacZ (1×10^{11} viral particles) were administered to six week old C57BL/6 female mice by tail vein injection and trachea installation. The animals were sacrificed and their liver and lung tissues were harvested at days 4, 9, 21, 28 and 35 post-administration. The transgene and viral late gene expression were compared.

At therapeutic doses of virus, there was diminished expression of late viral proteins at all time points in comparison with transgene.

C. Dose Responses in Liver

Dose responses of E4-deleted and E4 intact rAds in the liver of C57BL/6 mice were studied by tail vein administration of 1.5×10^{11} , 5×10^{10} , 1.7×10^{10} , 5.6×10^9 , and 1.9×10^9 viral particles and comparing the transgene and viral late gene expression at day 4, 21, 28, 35, and 42 post administration.

At therapeutic doses of virus, there was diminished expression of late viral proteins at all time points in comparison with transgene.

Example 9 - Other Gene Transfers

A. Human OTC Gene Transfer

The human OTC gene [A. L. Horwich et al, Science, 224:1068-174 (1984)] or the human CFTR gene [Riordan et al, Science, 245:1066-1073 (1989)] was used to replace the LacZ as the transgene in the recombinant E1/E4 deleted adenoviruses described above, using the techniques analogous for the construction of the above-

described *LacZ* vectors.

The resulting human OTC-containing rAd were administered at an MOI of 10 to 30 to human hepatocytes. The E1/E4 deleted rAd demonstrated less replication and less late gene expression than when the E1/E4 deleted rAds are administered to muscle, as described in the example above. However, the results of this gene transfer are better than comparable transfers with rAds containing only a deletion in the E1 gene or a deletion in the E1 gene and a point mutation in the E2a gene.

Similar results are demonstrated when the transgene is CFTR and the method of administration is intratracheal into lungs.

Example 10 - Transduction Efficiency of rAAV *LacZ* AV.CMVLacZ) in HeLa Cells Infected with Ad Mutants

A. Viruses

The following viruses were employed in this experiment:

- (1) Wild-type Ad 5, propagated in 293 cells;
- (2) Ad dl110 (an Ad which is deleted of the 55 kb E1B gene) [Babiss et al, *J. Virol.*, 52(2):389-395 (1984) and Babiss and Ginsberg, *J. Virol.*, 50(1):202-212 (1984)], propagated in 293 cells,
- (3) H5.CBALP (an Ad deleted of its E1A and E1B genes and containing a minigene that expresses alkaline phosphatase from a CMV enhanced β -actin promoter, as described above), propagated in 293 cells,
- (4) Ad ts125 (an Ad with a temperature sensitive mutation in the E2A gene which encodes the DNA binding protein) [Ensinger and Ginsberg, *J. Virol.*, 10(3):328-339 (1972)], propagated in 293 cells,
- (5) Ad dl802 (an Ad deleted of its E2a gene), grown in E2A-complementing gmDBP cells as described in Rice and Klessig, *J. Virol.*, 56(3):767-778 (1985);

(6) Ad dl1004 (an Ad deleted of the E4 gene), grown in E4-complementing Vero W162 cells [Weinberg and Ketner, Proc. Natl. Acad. Sci. USA, 80(17):5383-5386 (1983)] and

- 5 (7) Ad dl1010 (an Ad deleted of ORF6 of its E4 gene), grown in E4-complementing Vero W162 cells [Weinberg and Ketner, cited above].

All viruses were purified by two sequential rounds of buoyant density ultracentrifugation in CsCl.

10 B. *Experimental Procedures*

HeLa cells seeded in 6 well, 36mm culture plates (2×10^6 cells/well) were infected with wild-type Ad5 or an adenovirus early gene mutant as described in Part A at an MOI of 10pfu/well. Infections were done in 15 1.0 ml DMEM/2%FBS. Six hours post-infection, monolayers were washed and 1.0 ml fresh DMEM/2% FBS media containing AV.CMV LacZ at 4×10^9 virus particles/ml were added. Although the AV.CMV LacZ virus lot used in these experiments was shown to be free of H5.CLALP helper virus 20 by histochemical staining, the virus sample was subjected to heat treatment (60°C for 20 minutes) prior to use to ensure the absence of contaminating adenovirus. Two hours later, 1.0 ml of DMEM/115% FBS was added to each well.

25 Twenty-four hours after the addition of AV.CMV LacZ , cells were harvested. Each test condition was done in triplicate to enable virus transduction to be evaluated in terms of three outputs: histochemical staining for β -galactosidase activity (below), 30 intracellular β -galactosidase specific activity (Example 11), and the molecular form of the virus DNA (Example 12).

HeLa cells were histochemically stained for *E. coli* β -galactosidase (LacZ) activity as described in J. 35 M. Wilson et al, Proc. Natl. Acad. Sci. USA, 85:3014-3018

(1988). The different combinations that were tested included cells transfected with AAV vector alone (AV.CMVLacZ), vector plus wild-type Ad5 (+Ad5), vector plus dl110 (+dl110), vector plus Ad mutant H5.CBALP (+H5.CBALP), vector plus Ad mutant ts125 (+ts125), vector plus Ad mutant dl802 (+dl802), vector plus Ad mutant dl1004 (+dl1004), and vector plus Ad mutant dl1010 (+dl1010).

The results were observed in photomicrographs at magnification 10X (not pictured) of histochemical stains for recombinant β -galactosidase activity. The results indicated that wild-type Ad5 and the E2a mutants ts125 and dl802 caused a significant increase in LacZ rAAV transduction as measured by the number of positive blue cells and the degree of stain intensity. Both dl110 (E1B-55kDa) and dl1010 (ORF6) enhanced transduction to levels that approached those of Ad5, ts125, and dl802 in terms of the number of positive blue cells.

The E1 deletion recombinant H5.CBALP provided no significant increase in AV.CMVLacZ transduction. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction as evidenced by lack of significant increase in transduction obtained with HeLa cells infected with the E4 deletion mutant dl1004. A significant drop in transduction occurred following removal of ORF6 from the E4 region from the coinfecting adenovirus (Fig. 5A).

It is believed that these results demonstrate that the adenoviral gene products, E4 and E1 indirectly promote the formation of ds DNA intermediates that are transcriptionally active.

Example 11 - Quantitation of Enhanced Vector Transduction

(A) A duplicate set of HeLa cells as described in Example 10B were used in this experiment. Twenty-four hours after the addition of AV.CMVLacZ recombinant, for

intracellular β -galactosidase assays, cell pellets were suspended in 0.5 ml PBS and sonicated. Cell debris was removed by centrifugation (15,000Xg for 10 minutes) and the clarified extract assayed for total protein [M.

5 Bradford, Anal. Biochem., 72(1-2):248-254 (1976) and M. Bradford et al, Fed. Proc., 35(3):274 (1976)] and β -galactosidase activity [~~Sambrook et al, cited above~~] using o-nitrophenyl β -D-galactopyranoside (ONPG) as substrate.

10 Fig. 5A demonstrates the transduction efficiency quantitated by measuring β -galactosidase enzyme activity in the lysates from infected Hela cells and also assayed for total protein. In Fig. 5A, the test condition is shown along the horizontal axis, and
15 intracellular β -galactosidase specific activity (milliunits/mg protein) using ONPG as substrate is plotted on the vertical axis. Below each bar, the fold-induction in specific activity relative to cells that received the AV.CMVLacZ vector alone is given.

20 The results of Fig. 5A demonstrate that the E2a mutants ts125 and dl802 produced 134-fold and 225-fold increases in β -galactosidase activity, respectively, as compared to that achieved with purified rAAV alone. In comparison, cells infected with wt Ad5 generated 107-fold
25 increase in β -galactosidase activity.

(B) In another experiment, HeLa cells (2×10^6) were infected with increasing multiplicities of wild-type Ad5 or the E2 mutant dl802. Six hours post-infection, monolayers were washed and infected with AV.CMVLacZ at
30 1000 virus particles/cell. Twenty-four hours after the addition of AV.CMVLacZ, cells were harvested and assayed for total protein and β -galactosidase activity.

The results are illustrated in the bar graph of Fig. 5B, in which adenovirus MOI's are given along the
35 horizontal axis, and intracellular β -galactosidase

specific activity along the vertical axis. Enhancement of rAAV transduction was proportional to input helper adenovirus from MOIs of 1 to 50 for both wild type Ad5 and dl802. Higher doses of virus were cytopathic, leading to a fall in β -galactosidase expression. Enhanced transduction was achieved when the cells were infected prior to, or at the time of, rAAV infection. The E1 deletion recombinant H5.CBALP and the E4 deletion mutant dl1004 provided no significant increase in AV.CMVLacZ transduction. Both cells infected with dl110 (E1B-55kDa) and with dl1010 (ORF6) demonstrated substantially lower total β -galactosidase activity than those infected with Ad5, ts125, or dl802.

Example 12 - Analysis of Low Molecular Weight DNAs in AV.CMVLacZ Transduced Cells

Studies with these early gene mutants of adenovirus suggested that expression of adenoviral genes rather than the virion itself was responsible for enhancement of rAAV transduction. To further investigate these mechanisms and to determine if conversion of ss to ds genome limits the transduction efficiency of rAAV, the molecular state of the rAAV genome was characterized in the infected cells. The relationship between RFm formation and lacZ rAAV transduction was explored in experiments where the dose of coinfecting virus was varied (MOI=1, 5, or 10).

(A) A duplicate set of HeLa monolayers as described in Example 10 were harvested 24 hours after they were transduced with the recombinant AV.CMVLacZ and cultured with or without helper adenovirus.

Episomal DNA was extracted from cell pellets using a modification of the procedure originally described by B. Hirt, J. Mol. Biol., 26:365-369 (1967). Briefly, cells were suspended in 320 ml Tris-Cl (pH8.0)/10 mM EDTA and SDS added to a final concentration of 1%. The mixture was incubated at 37°C for 30 minutes.

Pronase and proteinase K were added to final concentrations of 500 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$, respectively, and the reaction incubated at 37°C for 2 hours. Sodium chloride was added to a final concentration of 1.1 M and
5 incubated at 4°C overnight. The precipitate that developed during the 4°C incubation was pelleted at 20,000 $\times g$ for 30 minutes and the clear supernatant carefully removed. The supernatant was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) followed
10 by chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with ethanol. The final pellet was suspended in 50 μl Tris-Cl (pH 8.0)/1.0 mM EDTA.

These Hirt extracts were analyzed by Southern blot hybridization. Samples (5 μl) of each Hirt extract
15 were resolved through a 1.2% agarose gel, electroblotted onto a nylon membrane and hybridized with a ^{32}P random primer labeled cDNA of the SV40 polyA signal used in AV.CMV LacZ .

An autoradiogram of the experiment of Example
20 12 (not pictured), identifies and labels bands corresponding to the ss AV.CMV LacZ genome (SS), a monomer replicative form (RFm), and concatomer replicative forms (RFd). Bands corresponding to the ss AV.CMV LacZ genome (SS), a monomer replicative form (RFm), and concatomer
25 replicative forms (RFd) were identified and labeled. To reference the RFm band, a plasmid carrying AV.CMV LacZ was digested to release the entire genome. Autoradiogram exposure times were 14 hours and 69 hours.

In this autoradiogram, the full spectrum of
30 molecular species present during a lytic infection was demonstrated in cells infected with both LacZ rAAV and wild type adenovirus. Both the input ss genome (SS) and monomeric and dimeric forms of ds replicative intermediates (RFm and RFd) are present. This contrasts
35 with cells infected with purified rAAV alone, where ss

genome is the sole molecular form detected. Analysis of cells coinfectd with the adenovirus early gene mutants revealed a direct correlation between formation of ds forms of the rAAV genome and the enhancement of LacZ transduction. Mutant adenoviruses that were ineffective in enhancing rAAV transduction (i.e., the E1 deleted mutant H5.CBALP and the E4 deleted mutant dl1004) failed to promote the formation of ds forms of AAV.

Cells infected with adenovirus deleted of E2a (dl802) or partially deleted of E1 (dl110) or E4 (dl1010) additionally demonstrated a band whose size was identical to the ds replicative monomer (RFm) of the lacZ rAAV genome and whose abundance correlated directly with the expression of β -galactosidase activity (compare results of Example 14 to these described results). Slower migrating concatomers, likely dimers, of duplex rAAV were also detected in the autoradiogram described above.

In the presence of E1 and E4 expression, rAd transduction was invariably accompanied by the appearance of ds RF monomers and dimers.

The high molecular weight band in sample lane +H5.CBALP is helper virus DNA. Helper virus DNA is recognized by the SV40 probe because the CBALP minigene also utilizes the SV40 polyA signal.

(B) In another experiment, HeLa cells were infected with wt Ad5 or the E2 deleted mutant dl802 as described in Example 10B. Monolayers were harvested 24 hours later and analyzed for β -galactosidase activity and RFm synthesis. Monomer bands similar to those shown in the autoradiogram described above were quantitated on a PhosphorImager 445 SI and assigned values (CPM).

The results are illustrated in the graphs of Figs. 8A and 8B, in which β -galactosidase specific activity and CPM are plotted along the vertical axis of each figure. Adenovirus MOI's are given on the horizontal

axis of each figure. Data obtained from low MOI infections (1, 5, and 10) are shown. Importantly, the tight correlation between rAAV vector transduction and the accumulation of duplex forms could be achieved in cells infected with E1/E4 expressing adenovirus. The level of β -galactosidase and abundance of RFm increased in proportion to the amount of infecting wild-type Ad (Fig. 6A) and dl802 (Fig 6B). These data suggest that synthesis of an episomal duplex intermediate is an obligatory event in transduction.

Example 13 - Duplex End-Analysis

The following is a description of a model for leading strand synthesis of a complementary AAV strand in the presence of Rep (+Rep) or absence of Rep (-Rep). Refer to Figs. 7A-7F. Rep expresses a terminal resolution activity that can convert a duplex structure with closed-ends to an open-ended duplex. In the absence of Rep, terminal resolution is impaired leaving the covalently closed, hairpin structures intact. Under these conditions, hairpins are expected to be found leftward and rightward, since both strands of a rescued ds AAV genome are packaged into virions. Figs. 7B-7F are a flow chart demonstrating the strategy for identifying the terminal structure of duplex RFm that is synthesized from ss AV.CMV LacZ in response to adenoviral gene expression.

Fig. 7C illustrates a closed end and an open end fragment of rAV.CMV LacZ . Figs. 7D, 7E and 7F indicate the mixture of open-ended and covalently closed duplex fragments generated by NotI digestion at position 4509 in the absence of terminal resolution. The NotI 4509 digestion provides a convenient means of releasing a 361 bp fragment that contains the right ITR in the context of a hybridization target (i.e. SV40 pA). In the presence of terminal resolution, only the open-ended 361 bp

fragment would be expected to be generated (Fig. 7D) by such digestion.

The resulting electrophoretic gel (not pictured), revealed in lane (1) the results of digestion of a
5 plasmid carrying an AV.CMVLacZ cDNA to release the rAAV vector, and subsequent digestion with NotI to release the right terminal 361 bp fragment. In lane (2) a sample of
NotI digested Hirt DNA extracted from HeLa cells infected with wild-type Ad5 and transduced with AV.CMVLacZ
10 resulted in the release of two fragments, labeled FormI and FormII. (See, also, Figs. 8A and 8B). The migration of ss AV.CMVLacZ (SS) and RFm were also seen.

The ds AV.CMVLacZ intermediates that accumulated in cells infected with adenovirus were likely the result of
15 leading strand DNA synthesis, initiating from the duplex region of the vector ITR. In the absence of Rep, this conversion event was anticipated to generate molecules in which one end is open and the other is covalently closed (Fig. 7A). To further characterize the structure of this
20 ds intermediate Hirt extracts from cells coinfectd with rAV.CMVLacZ and Ad5 were digested with NotI to release the termini of the ds intermediate which, if left open, would be approximately 361 bp in length. The resulting filters were hybridized with a probe specific for the
25 SV40 polyadenylation signal positioned immediately upstream of the rightward ITR. At least two forms were released from the right end of duplex genomes, one that migrated to a position in the gel that predicted an open-ended conformation (Form II), and a second slower
30 migrating species (Form I). Although this result was consistent with the model (Figs. 7A-7F), it was difficult to predict with certainty the structure of Form I. Its retarded mobility did, however, suggest a conformation that differed from the open-ended Form II.

Example 14 - Analysis of AV.CMV_{LacZ} Transduction
Efficiency in 293 Cells Stably Transfected with an
Inducible E4 ORF6 cDNA

Cell lines used in this assay were prepared as described in Example 1. 293(MT-ORF6) cells and HeLa(MT-ORF6) cells were seeded in 6 well 35 mm culture plates (2x10⁶ cells/well) and infected with purified, heat-treated AV.CMV_{LacZ} at an MOI of 1000 virus particles/cell. Induction of ORF6 expression with from none to increasing concentrations of zinc sulfate was initiated 2 hours before the addition of virus and continued throughout the duration of the experiment.

Twenty-four hours after the addition of virus, cells were harvested, lysates were generated by sonication and analyzed for the β -galactosidase expression (i.e., β -galactosidase activity) and virus DNA as described in the preceding examples. Hirt extracts were prepared from low molecular weight DNA from cell extracts. The preparation of the Hirt extracts and subsequent analysis by Southern hybridization were performed similarly to those described in the examples above.

The results of this experiment were as follows:

(1) Specific Activity

The results are illustrated in the bar graph of Fig. 8A. Specific activity (milliunits β -galactosidase/mg protein) is plotted along the vertical axis. Below each bar is given the concentration of zinc used for induction, the fold-induction relative to 293 cells, and the fold-induction relative to 293(ORF6) cells maintained in the absence of zinc. As shown in Fig. 8A, in the absence of Zn⁺², the 293(MT-ORF6) cell line generated 39-fold higher levels of β -galactosidase in rAAV infected 293 cells. Induction of ORF6 expression with increasing amounts of Zn⁺² resulted in a concomitant rise in AV.CMV_{LacZ} cell transduction to a level that was

445-fold greater than the parent 293 line. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction.

The specific activity of β -galactosidase was 196.2 mUnits/mg in E1/E4 expressing 293 cells, compared to 1.0 mUnit/mg in 293 cells that only expressed E1 genes. These experiments support a mechanism for enhancing rAAV transduction that is dependent on the combined expression of both E1 and E4 adenoviral genes.

(2) Molecular Analysis of the AV.CMV β LacZ Genome

The duplex monomer replicative form (RFm) was quantitated and the values (CPM) plotted along the vertical axis in the bar graph of Fig. 8B. The concentration of zinc used for induction and the fold-induction relative to 293(ORF6) cells maintained in 0 mM zinc is given below each bar.

An autoradiogram (not pictured) shows the agarose gel resolved Hirt extracts from the AV.CMV β LacZ transduced cells described above. A plasmid carrying the AV.CMV β LacZ cDNA was digested to release the entire sequence and loaded in a lane of the autoradiogram. The band that appeared in this lane therefore reflected the migration of a monomer duplex replicative form (RFm). The migration of the ss AV.CMV β LacZ genome (SS), RFm, and dimers of the duplex replicative form (RFd) were also shown. Lanes of the autoradiogram labeled (0), (50), (100), (150), (200), and (250) contained samples from 293(MT-ORF6) cells that were induced with the indicated concentration of zinc. A Hirt extract from 293 cells (lane labeled 293) transduced with AV.CMV β LacZ was also shown.

Analysis of Hirt extracts revealed the presence of the RFm in the rAAV infected 293(MT-ORF6) cells that was not present in similarly infected 293

cells. When the induction profiles (Figs. 8A and 8B) that describe AV.CMVLacZ transduction efficiency were compared, the results were plotted in Fig. 8C. Specific activity (milliunits β -galactosidase/mg protein) data from Fig. 8A and counts-per-minute data (CPM) of AV.CMVLacZ Rfm from Fig. 8B are plotted along the vertical axis, and concentration of zinc sulfate used during the experiment is shown along the horizontal axis.

The two profiles are near mirror images. Importantly, the Rfm increased in proportion to the increment in lacZ transducing activity that occurred as ORF-6 expression was induced with Zn^{+2} (Fig. 8C). Similar results were obtained with a 293 derived cell line that expresses ORF6 from the glucocorticoid responsive MMTV promoter.

Example 15 - Enhanced AV.CMVLacZ Transduction in HeLa Cells Carrying an Inducible ORF6 Minigene

HeLa(MT-ORF6) cells (2×10^6) were transduced at an MOI of 1,000 AV.CMVLacZ recombinant particles/cell in absence of zinc sulfate inducer or in the presence of 50, 100, 150, 200, or 250 μ M zinc sulfate inducer in the media during transduction. Twenty-four hours later, cells were harvested, cell extracts were prepared by sonication, and analyzed for transgene expression (i.e., β -galactosidase activity). Cell monolayers were histochemically stained for β -galactosidase activity.

The resulting photomicrographs (not pictured) illustrated that histochemical staining revealed an increase in the number of cells scored lacZ positive as the concentration of Zn^{+2} in the medium was raised from 0 to 200 mM. Concentrations of 250 mM zinc were found to be toxic to the cells.

Specific activity (milliunits β -galactosidase/mg protein) is plotted in Fig. 9 along the vertical axis. Below each bar is given the concentration of zinc used

for induction, the fold-induction relative to HeLa cells, and the fold-induction relative to HeLa(MT-ORF6) cells maintained in the absence of zinc. Histochemical staining revealed an increase in the amount of

5 β -galactosidase in lysates as the concentration of Zn^{+2} in the medium was raised from 0 to 200 mM.

Example 16 - Southern Blot Analysis of Low Molecular Weight DNAs from AV.CMVLacZ Transduced HeLa(MT-ORF6) Cells

10 Following Induction of E4ORF6

Hirt extracts were prepared from HeLa(MT-ORF6) cells transduced with AV.CMVLacZ as described in Example 15 in the presence of increasing concentrations of Zn^{+2} to determine whether synthesis of duplex intermediates

15 contributed to the augmentation in AV.CMVLacZ transduction.

Samples of HeLa(MT-ORF6) cells that were induced with a concentration of zinc sulfate (0, 50, 100, 150, 200, and 250) were resolved on a 1.2% agarose Southern

20 gel (not pictured), transferred to a nylon membrane, and hybridized with a LacZ-specific probe. One lane contained a plasmid encoding AV.CMVLacZ that was digested to release the entire genome. Bands corresponding to the ss AV.CMVLacZ genome (SS), duplex monomers (RFm), and

25 duplex dimers (Rfd) were indicated on the gel.

Southern analysis indicated that HeLa and uninduced HeLa(MT-ORF6) cells demonstrated a single band on Southern blots which comigrated with the ss genome. Induction of ORF-6 resulted in the appearance of

30 detectable levels of ds monomer but only at higher concentrations of Zn^{+2} . A band comigrating with the Rfd was present in all cell preparations, the relevance of which is unclear since the monomer is a likely precursor to the dimer.

Example 17 - Effect of Adenovirus Infection on In Vivo
AV.CMVLacZ Targeting Efficiency To Murine Liver

The impact of adenoviral gene expression on rAAV transduction in murine liver was studied by sequentially
5 infusing into the portal vein early gene mutants of adenovirus followed by rAAV.

Balb/c mice, 4- to 6-weeks old [Jackson Laboratories, Bar Harbor, Maine] were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg) and
10 xylazine (10 mg/kg). For liver studies, a 1 cm left flank incision was made and the spleen exposed.

Samples of purified, heat-treated AV.CMVLacZ in 50 μ l HBS (1×10^{11} virus particles) were used alone or spiked with helper adenovirus containing 2×10^{10} A_{260} particles of
15 purified dl1004, H5.CBALP, or ts125 in a final volume of 50 μ l. The dose of adenovirus was sufficient to transduce >25% of hepatocytes. The virus mixture was injected just beneath the splenic capsule and the abdomen was closed with 3-0 vicryl.

20 Necropsies were performed 3 days post-infusion and tissue frozen in O.C.T. embedding compound. Frozen sections (6 μ m) (LacZ+ALP) were prepared and histochemically stained for β -galactosidase enzyme and alkaline phosphatase activity. Sections were
25 counterstained with neutral red and mounted.

A β -galactosidase positive hepatocyte targeted with AV.CMVLacZ at magnification 20X was obtained. Histochemical analyses of liver tissue harvested 3 days after gene transfer demonstrated that administration of
30 10^{11} particles of purified rAV.CMVLacZ alone into the portal vein was not associated with appreciable gene transfer (<0.01% of cells), confirming the inherent inefficiency of the rAAV system.

Preinfusion with E4 deleted virus had no impact on
35 rAAV transduction in mouse liver, whereas E1 deleted

virus demonstrated a modest increment in *lacZ* positive hepatocytes to about 0.1%. The most significant increase in rAAV transduction occurred following infusion of the E2a adenovirus mutant ts125 with *lacZ* expression detected in 10-25% of hepatocytes. A direct relationship between adenovirus gene expression and rAAV transduction was demonstrated in animals infused with both *lacZ*-rAAV and the ALP expressing E1 deleted virus. The dose of adenovirus was reduced 10-fold to minimize the coincidental occurrence of coinfection. Histochemical studies demonstrated co-localization of ALP and β -galactosidase in the majority of β -galactosidase expressing hepatocytes.

Example 18 - Effect of Adenovirus Infection on In Vivo AV.CMVLacZ Targeting Efficiency To Murine Lung

Experiments described in Example 17 for mouse liver were adapted for the study of rAAV mediated gene transfer to mouse lung. For lung experiments, anesthetized Balb/C animals were intubated as described in DeMatteo et al, Transplantation (Baltimore), 59(5):787-789 (1995). Briefly, a midline 2 cm skin incision was made in the neck to expose the trachea. A 2 inch 18 gauge angiocatheter was passed through the mouth, positioned in the midportion of the trachea, and connected to a rodent ventilator (#55-3438 Harvard). Polyethylene (PE#10, Intramedic) was fed through the catheter via a side port and advanced beyond the tracheal bifurcation. Using a Hamilton syringe, virus samples (30 μ l) were slowly infused into the lung through the polyethylene tubing. Samples contained the same formulation of purified, heat-treated AV.CMVLacZ with or without helper adenovirus, as described for liver injections.

Tissue was harvested 72 hours post-infusion. Frozen sections were histochemically stained for β -galactosidase activity and counterstained with neutral red.

Frozen sections from lung (AV.CMVLacZ) showed a β -galactosidase positive airway epithelial cell targeted with AV.CMVLacZ. Similar studies were performed in the murine model of lung-directed gene transfer.

- 5 Adenoviruses were instilled into the trachea prior to the instillation of rAAV. Analysis of lung tissue 3 days later revealed only a rare β -galactosidase positive cell in animals instilled with rAAV alone. No detectable enhancement of rAAV transduction was noted in animals
10 preinstilled with adenovirus deleted of either E1 or E4. Substantial enhancement of transduction was achieved in conducting airway and alveolar cells of animals administered the E2a mutant adenovirus.

- These experiments in murine models of gene therapy
15 directed to liver and lung verified that the efficiency of rAAV transduction is low due limited conversion of the input ss genome to a transcriptionally active ds intermediate, and that this conversion is facilitated by expression of adenovirus E1 and E4 gene products.

20 Example 19 - Second Generation rAAV with Regulated Minigene Capable of Enhancing Transduction

- The experiments described in previous examples illustrated the following principles: 1) purified rAAV is a relatively inefficient gene transfer vehicle *in vitro*
25 and *in vivo* and 2) the rate limiting step in transduction is not viral entry but rather conversion of the virion's ss DNA genome to a transcriptionally active ds DNA genome. Adenovirus can substantially enhance transduction through expression of a subset of its genes.
30 It does this by promoting conversion of the virion's genome to its ds form. One approach to accomplish this is to incorporate into the recombinant AAV genome a minigene that expresses the minimal adenoviral genes necessary to enhance transduction, i.e., the ORF6 region
35 of E4.

Two approaches have been considered in designing this modified rAAV. The first strategy is based on a rAAV genome that has two transcriptional units in series, one expressing the therapeutic gene and the other
5 expressing its E4 ORF6 from a constitutive promoter. While this may, in fact, be useful in many situations, ~~constitutive expression of ORF6 may be detrimental to the~~ cell and potentially could elicit a destructive immune response.

10 The second version of this rAAV includes the therapeutic minigene in addition to the ORF6 transcriptional unit which, in this case, is expressed from an inducible promoter. When this second gene rAAV is administered to the cells (*ex vivo* strategies) or to
15 the patient (*in vivo* strategies), the inducing agents are administered at the time of gene transfer or soon thereafter. If the ds genomic form or its integrated derivative is stable, the induction of ORF6 will only be necessary at the time of gene transfer into the recipient
20 cell. Following this, its inducing agent will be withdrawn and the ORF6 gene will be turned off.

An rAAV that illustrates this concept of inducible ORF6 has been constructed and tested *in vitro*. A schematic of the vector pAV.CMVALP.GRE-ORF6, is shown in
25 Fig. 11 and its sequence is illustrated in SEQ ID NO: 5. This second generation construct contains flanking 5' and 3' AAV ITR sequences. The human placental alkaline phosphatase cDNA (ALP) is included in a minigene in which the promoter from the immediate early gene of
30 cytomegalovirus drives the transcription. A second transcriptional unit is cloned between the ITRs in series and in direct orientation with the alkaline phosphatase minigene. The second transcriptional unit expresses the Ad5-E4-ORF6 from a glucocorticoid dependent promoter
35 (GRE) with an SV40 polyadenylation signal. This is

called a second generation rAAV construct.

Specifically, pAV.CMVALP.GRE-ORF6 [SEQ ID NO: 5] generates a novel rAAV containing the *LacZ* transgene and the Ad E4 ORF 6 which facilitates ss to ds conversion of rAAV. The plasmid includes a flanking AAV 5' ITR sequence (nucs. 53-219); CMV enhancer/promoter (nucs. 255-848); ~~human placenta alkaline phosphatase cDNA (ALP)~~ (nucs. 914-2892); SV40 polyA (nucs. 2893-3090); GRE promoter (nucs. 3114-3393); Ad5 E4-ORF6 cDNA (nucs. 3402-4286); SV40 polyA (nucs. 4315-4512); and 3' AAV ITR (nucs. 4547-4713). All other nucleotides are plasmid derived.

The second generation rAAV construct was used to produce and purify rAAV virions which were exposed to HeLa cells that were left untreated or incubated with dexamethasone. In the absence of dexamethasone, (a condition under which little ORF6 should be expressed), little transduction was observed as measured by expression of the alkaline phosphatase gene. Cells incubated in dexamethasone expressed in ORF6 gene and the transduction efficacy was enhanced at least 5-fold. This provides evidence to support that a gene product expressed from the rAAV can function in cis to enhance expression of the transgene.

Example 20 - Application to Bone Marrow Directed Gene Therapy

Bone marrow directed gene therapy represents the paradigm of ex vivo gene therapy where the target cell is the hematopoietic stem cell. The basic strategy is to incorporate (i.e., integrate) a therapeutic minigene into the chromosomal DNA of hematopoietic stem cells which are transplanted into a recipient patient whose own bone marrow has been ablated allowing repopulation of its lymphohematopoietic system with progeny of the genetically corrected stem cell.

The problem with this approach has been efficiently transfecting genes into stem cells. Most studies of bone marrow directed gene therapy have utilized recombinant retroviruses which have not been very efficient. One
5 problem is that retroviruses integrate their provirus only when the target cell is dividing. Unfortunately, ~~most stem cells in vitro are quiescent and not dividing.~~ rAAV holds the promise of integrating the provirus more efficiently into non-dividing stem cells. However,
10 purified rAAV is not very efficient with respect to integration when used alone. In cultured cells, integration is observed in less than 1% of the cells. The same conditions that activate the conversion of ss to ds genome also enhance the integration of the ds
15 intermediate into the chromosomal DNA.

Therefore, a desirable application of the methods and compositions of this invention is in bone marrow directed gene therapy. According to this method, stem cells are genetically modified with rAAV and an inducing
20 agent ex vivo using the constructs and methods described above (see e.g., Example 19). Genetically modified stem cells are subsequently transplanted by conventional techniques.

Numerous modifications and variations of the present
25 invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as selections of different transgenes and plasmids
30 for the construction of the packaging cell lines and rAds, or selection or dosage of the viruses or immune modulators, are believed to be encompassed in the scope of the claims appended hereto.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of the University of Pennsylvania
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Fisher, Krishna J.
Gao, Guang-Ping

(ii) ~~TITLE OF INVENTION: Recombinant Adenovirus and Adeno-~~
Associated Virus, Cell Lines,
and Methods of Production
and Use Thereof

(iii) NUMBER OF SEQUENCES: 5

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(B) COMPUTER: IBM PC compatible
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(D) SOFTWARE: PatentIn Release 1.0 Version 1.30

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3653 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1521..2405

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCATGTGT CAGAGGTTTT CACCGTCATC ACCGAAACGC GCGAGGCAGC	50
AAGCTTGGCA GAAATGGTTG AACTCCCGAG AGTGTCTTAC ACCTAGGGGA	100
GAAGCAGCCA AGGGGTTGTT TCCCACCAAG GACGACCCGT CTGCGCACAA	150
ACGGATGAGC CCATCAGACA AAGACATATT CATTCTCTGC TGCAAACCTG	200
GCATAGCTCT GCTTTGCCTG GGGCTATTGG GGGAAGTTGC GGTTCGTGCT	250
CGCAGGGGCTC TCACCCTTGA CTCTTTCAAT AATAACTCTT CTGTGCAAGA	300
TTACAATCTA AACAAATTCGG AGAACTCGAC CTTCTCCTG AGGCAAGGAC	350
CACAGCCAAC TTCCTCTTAC AAGCCGCATC GATTTTGTCC TTCAGAAATA	400
GAAATAAGAA TGCTTGCTAA AAATTATATT TTTACCAATA AGACCAATCC	450
AATAGGTAGA TTATTAGTTA CTATGTTAAG AAATGAATCA TTATCTTTTA	500
GTACTATTTT TACTCAAATT CAGAAGTTAG AAATGGGAAT AGAAAAATAGA	550
AAGAGACGCT CAACCTCAAT TGAAGAACAG GTGCAAGGAC TATTGACCAC	600
AGGCCTAGAA GTAAAAAAGG GAAAAAAGAG TGTTTTTGTG AAAATAGGAG	650
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ACAGATGCCC CCTTACCATA TACAGGAAGA TATGACTTAA ATTGGGATAG	750
GTGGGTTACA GTCAATGGCT ATAAAGTGTT ATATAGATCC CTCCCCTTTC	800
GTGAAAGACT CGCCAGAGCT AGACCTCCTT GGTGTATGTT GTCTCAAGAA	850
AAGAAAGACG ACATGAAACA ACAGGTACAT GATTATATTT ATCTAGGAAC	900

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AGGAATGCAC	TTTTGGGGAA	AGATTTTCCA	TACCAAGGAG	GGGACAGTGG	950
CTGGACTAAT	AGAACATTAT	TCTGCAAAAA	CTTATGGCAT	GAGTTATTAT	1000
GATTAGCCTT	GATTTGCCCA	ACCTTGCGGT	TCCCAAGGCT	TAAGTAAGTT	1050
TTTGGTTACA	AACTGTTCTT	AAAACAAGGA	TGTGAGACAA	GTGGTTTCCT	1100
GACTTGGTTT	GGTATCAAAG	GTTCTGATCT	GAGCTCTGAG	TGTTCTATTT	1150
TCCTATGTTT	TTTTGGAATT	TATCCAAATC	TTATGTAAAT	GCTTATGTAA	1200
ACCAAGATAT	AAAAGAGTGC	TGATTTTTTG	AGTAAACTTG	CAACAGTCCT	1250
AACATTCACC	TCTTGTGTGT	TTGTGTCTGT	TCGCCATCCC	GTCTCCGCTC	1300
GTCACCTATC	CTTCACCTTC	CAGAGGGTCC	CCCCGCAGAC	CCCGGCGACC	1350
CTCAGGTCGG	CCGACTGCGG	CAGCTGGCGC	CCGAACAGGG	ACCCTCGGAT	1400
AAGTGACCCT	TGTCTTTATT	TCTACTATTT	TGTGTTTCGT	TTGTTTTGTC	1450
TCTATCTTGT	CTGGCTATCA	TCACAAGAGC	GGAACGGACT	CACCTCAGGG	1500
AACCAAGCTA	GCCCAATTCT	ATGACTACGT	CCGGCGTTCC	ATTTGGCATG	1550
ACACTACGAC	CAACACGATC	TCGGTTGTCT	CGGCGCACTC	CGTACAGTAG	1600
GGATCGTCTA	CCTCCTTTTG	AGACAGAAAC	CCGCGCTACC	ATACTGGAGG	1650
ATCATCCGCT	GCTGCCCGAA	TGTAACACTT	TGACAATGCA	CAACGTGAGT	1700
TACGTGCGAG	GTCTTCCCTG	CAGTGTGGGA	TTACGCTGA	TTCAGGAATG	1750
GGTTGTTCCC	TGGGATATGG	TTCTAACGCG	GGAGGAGCTT	GTAATCCTGA	1800
GGAAGTGAT	GCACGTGTGC	CTGTGTTGTG	CCAACATTGA	TATCATGACG	1850
AGCATGATGA	TCCATGGTTA	CGAGTCCTGG	GCTCTCCACT	GTCATTGTTC	1900
CAGTCCCCGT	TCCCTGCAGT	GTATAGCCGG	CGGGCAGGTT	TTGGCCAGCT	1950
GGTTTAGGAT	GGTGGTGGAT	GGCGCCATGT	TTAATCAGAG	GTTTATATGG	2000
TACCGGGAGG	TGGTGAATTA	CAACATGCCA	AAAGAGGTAA	TGTTTATGTC	2050
CAGCGTGTTT	ATGAGGGGTC	GCCACTTAAT	CTACCTGCGC	TTGTGGTATG	2100
ATGGCCACGT	GGGTTCTGTG	GTCCCCGCCA	TGAGCTTTGG	ATACAGCGCC	2150
TTGCACTGTG	GGATTTTGAA	CAATATTGTG	GTGCTGTGCT	GCAGTTACTG	2200

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TGCTGATTTA	AGTGAGATCA	GGGTGCGCTG	CTGTGCCCCG	AGGACAAGGC	2250
GCCTTATGCT	GCGGGCGGTG	CGAATCATCG	CTGAGGAGAC	CACTGCCATG	2300
TTGTATTCCCT	GCAGGACGGA	GCGGCGGCGG	CAGCAGTTTA	TTGCGCGCT	2350
GCTGCAGCAC	CACCGCCCTA	TCCTGATGCA	CGATTATGAC	TCTACCCCCA	2400
TGTAGGGATC	CAAGCTTGCG	GGCGCATCGA	TGATATCAAG	CTTGCATGCC	2450
TGCAGGTCGA	CTCTAGAGGA	TCCCGGGTGG	NATCCCTGTG	ACCCCTCCCC	2500
AGTGCCTCTC	CTGGCCCTGG	AAGTTGGCAC	TCCAGTGCCC	ACCAGCCTTG	2550
TCCTAATAAA	ATTAAGTTGN	ATCATTTTGT	CTGACTAGGT	GTCCTTCTAT	2600
AATATTATGG	GGTGGAGGGG	GGTGGTATGG	AGCAANGGGN	AANTTGGNAA	2650
GACAANCTGT	AGGGCCTGCG	GGGTCTATTG	GGAACAAGCT	GGAGTGCAGT	2700
GGCACAATCT	TGGCTCACTG	CAATCTCCGC	CTCCTGGGTT	CAAGCGATTC	2750
TCCTGCCTCA	GACTCCCGAG	TTGTTGGGAT	TCCAGGCATG	CATGACCAGG	2800
CTCAGATAAT	TTTTGTTTTT	TTGGTAGAGA	CGGGGTTTCA	CCATATTGGN	2850
CAGGCTGGTC	TCCAACCTCT	AATCTCAGGT	GATCTNCCCA	CCTTGGCCTC	2900
CCAAATTGCT	GGGATTACAG	GNGTGAACCA	CTGNTCCCTT	CCCTGTCCTT	2950
CTGATTTTAA	AATAACTATA	CCAGCAGGAG	GACGTCCAGA	CACAGCATAG	3000
GCTACCTGGC	CATGCCCAAC	CGGTGGGACA	TTTGAGTTGC	TTGCTTGGCA	3050
CTGTCCTCTC	ATGCGTTGGG	TCCACTCAGT	AGATGCCTGT	TGAATTGGGT	3100
ACGCGGCCAG	CTTGGCTGTG	GAATGTGTGT	CAGTTAGGGT	GTGGAAAGTC	3150
CCCAGGCTCC	CCAGCAGGCA	GAAGTATGCA	AAGCATGCAT	CTCAATTAGT	3200
CAGCAACCAG	GTGTGGAAAG	TCCCCAGGCT	CCCCAGCAGG	CAGAAGTATG	3250
CAAAGCATGC	ATCTCAATTA	GTCAGNAACC	ATAGNCCCGC	CCCTAACTCC	3300
GTCCATCCCG	GCCCTAACTC	NGGCCAGTTC	CGACCNTNCT	CCGGCNNATG	3350
GNTGAGTAAT	TTGCNNGATT	TATGCAGNGG	GCGAGGNCGC	CTCGGGCTCT	3400
GAGNTNTTCC	AGAAGTAGTG	AGGAGGCTTT	NNTGGTGGAA	TTGATCAGCT	3450
TGGGATCTGA	TCAAGAGACA	GGATGAGGAT	CGNNNCGNAT	GATTGAACAA	3500

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GATGGGTTGC ACGGAGGTTC TCCGGNCGCT TGGGTGGGGA GGNTATTCGG 3550
 NTATTNTTGG TGNACAACAG NNAAACGGNT GTTCTGATGC CGCCGCGTTC 3600
 NCGCTTTCAG NGCAGGGGGG CCCCCCTTCT NTTGAGANNA GCNCCCCTTN 3650
 TTG 3653

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 294 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Thr	Thr	Ser	Gly	Val	Pro	Phe	Gly	Met	Thr	Leu	Arg	Pro	Thr	Arg	1	5	10	15
Ser	Arg	Leu	Ser	Arg	Arg	Thr	Pro	Tyr	Ser	Arg	Asp	Arg	Leu	Pro	Pro	20	25	30	
Phe	Glu	Thr	Glu	Thr	Arg	Ala	Thr	Ile	Leu	Glu	Asp	His	Pro	Leu	Leu	35	40	45	
Pro	Glu	Cys	Asn	Thr	Leu	Thr	Met	His	Asn	Val	Ser	Tyr	Val	Arg	Gly	50	55	60	
Leu	Pro	Cys	Ser	Val	Gly	Phe	Thr	Leu	Ile	Gln	Glu	Trp	Val	Val	Pro	65	70	75	80
Trp	Asp	Met	Val	Leu	Thr	Arg	Glu	Glu	Leu	Val	Ile	Leu	Arg	Lys	Cys	85	90	95	
Met	His	Val	Cys	Leu	Cys	Cys	Ala	Asn	Ile	Asp	Ile	Met	Thr	Ser	Met	100	105	110	
Met	Ile	Tyr	Gly	Tyr	Glu	Ser	Trp	Ala	Leu	His	Cys	His	Cys	Ser	Ser	115	120	125	
Pro	Gly	Ser	Leu	Gln	Cys	Ile	Ala	Gly	Gly	Gln	Val	Leu	Ala	Ser	Trp	130	135	140	
Phe	Arg	Met	Val	Val	Asp	Gly	Ala	Met	Phe	Asn	Gln	Arg	Phe	Ile	Trp	145	150	155	160

[illegible]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35408 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATCATCAAT	AATATACCTT	ATTTTGGATT	GAAGCCAATA	TGATAATGAG	50
GGGGTGGAGT	TTGTGACGTG	GCGCGGGGCG	TGGGAACGGG	GCGGGTGACG	100
TAGTAGTGTG	GCGGAAAGTGT	GATGTTGCAA	GTGTGGCGGA	ACACATGTAA	150
GCGACGGATG	TGGCAAAAGT	GACGTTTTTG	GTGTGCGCCG	GTGTACACAG	200
GAAGTGACAA	TTTTCGCGCG	GTTTTAGGCG	GATGTTGTAG	TAAATTG	250
CGTAACCGAG	TAAGATTTGG	CCATTTTCGC	GGGAAAAC	TGATAAGAGGA	300

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AGTGAAATCT	GAATAATTTT	GTGTACTCA	TAGCGCGTAA	TATTTGTCTA	350
GGGAGATCAG	CCTGCAGGTC	GTTACATAAC	TTACGGTAAA	TGGCCCCGCT	400
GGCTGACCGC	CCAACGACCC	CCGCCCATTG	ACGTCAATAA	TGACGTATGT	450
TCCCATAGTA	ACGCCAATAG	GGACTTTCCA	TTGACGTCAA	TGGGTGGAGT	500
ATTTACGGTA	AACTGCCCCAC	TTGGCAGTAC	ATCAAGTGTA	TCATATGCCA	550
AGTACGCCCC	CTATTGACGT	CAATGACGGT	AAATGGCCCCG	CCTGGCATT	600
TGCCCAGTAC	ATGACCTTAT	GGGACTTTCC	TACTTGGCAG	TACATCTACT	650
CGAGGCCACG	TTCTGCTTCA	CTCTCCCCAT	CTCCCCCCCC	TCCCCACCCC	700
CAATTTTGTA	TTTATTTATT	TTTAAATTAT	TTTGTGCAGC	GATGGGGGCG	750
GGGGGGGGGG	GGGGGCGCGC	GCCAGGCGGG	GCGGGGCGGG	GCGAGGGGCG	800
GGGCGGGGCG	AGGCGGAGAG	GTGCGGCGGC	AGCCAATCAG	AGCGGCGCGC	850
TCCGAAAGTT	TCCTTTTATG	GCGAGGCGGC	GGCGGCGGCG	GCCCTATAAA	900
AAGCGAAGCG	CGCGGCGGGC	GGGAGCGGGA	TCAGCCACCG	CGGTGGCGGC	950
CGCAATTCCC	GGGGATCGAA	AGAGCCTGCT	AAAGCAAAAA	AGAAGTCACC	1000
ATGTCGTTTA	CTTTGACCAA	CAAGAACGTG	ATTTTCGTTG	CCGGTCTGGG	1050
AGGCATTGGT	CTGGACACCA	GCAAGGAGCT	GCTCAAGCGC	GATCCCGTCG	1100
TTTTACAACG	TCGTGACTGG	GAAAACCCTG	GCGTTACCCA	ACTTAATCGC	1150
CTTGACGAC	ATCCCCCTTT	CGCCAGCTGG	CGTAATAGCG	AAGAGGCCCCG	1200
CACCGATCGC	CCTTCCCAAC	AGTTGCGCAG	CCTGAATGGC	GAATGGCGCT	1250
TTGCCTGGTT	TCCGGCACCA	GAAGCGGTGC	CGGAAAGCTG	GCTGGAGTGC	1300
GATCTTCCTG	AGGCCGATAC	TGTCGTCGTC	CCCTCAAAC	GGCAGATGCA	1350
CGGTTACGAT	GCGCCCATCT	ACACCAACGT	AACCTATCCC	ATTACGGTCA	1400
ATCCGCCGTT	TGTTCCCACG	GAGAATCCGA	CGGGTTGTTA	CTCGCTCACA	1450
TTTAATGTTG	ATGAAAGCTG	GCTACAGGAA	GGCCAGACGC	GAATTATTTT	1500
TGATGGCGTT	AACTCGGCGT	TTCATCTGTG	GTGCAACGGG	CGCTGGGTCTG	1550
GTTACGGCCA	GGACAGTCGT	TTGCCGTCTG	AATTTGACCT	GAGCGCATTT	1600

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TTACGCGCCG	GAGAAAACCG	CCTCGCGGTG	ATGGTGCTGC	GTTGGAGTGA	1650
CGGCAGTTAT	CTGGAAGATC	AGGATATGTG	GCGGATGAGC	GGCATTTTCC	1700
GTGACGTCTC	GTTGCTGCAT	AAACCGACTA	CACAAATCAG	CGATTTCCAT	1750
GTTGCCACTC	GCTTTAATGA	TGATTTTCAGC	CGCGCTGTAC	TGGAGGCTGA	1800
AGTTCAGATG	TGCGGCGAGT	TGCGTGACTA	CCTACGGGTA	ACAGTTTCTT	1850
TATGGCAGGG	TGAAACGCAG	GTCGCCAGCG	GCACCGCGCC	TTTCGGCGGT	1900
GAAATTATCG	ATGAGCGTGG	TGGTTATGCC	GATCGCGTCA	CACTACGTCT	1950
GAACGTCGAA	AACCCGAAAC	TGTGGAGCGC	CGAAATCCCG	AATCTCTATC	2000
GTGCGGTGGT	TGAACTGCAC	ACCGCCGACG	GCACGCTGAT	TGAAGCAGAA	2050
GCCTGCGATG	TCGGTTTCCG	CGAGGTGCGG	ATTGAAAATG	GTCTGCTGCT	2100
GCTGAACGGC	AAGCCGTTGC	TGATTCGAGG	CGTTAACCGT	CACGAGCATC	2150
ATCCTCTGCA	TGGTCAGGTC	ATGGATGAGC	AGACGATGGT	GCAGGATATC	2200
CTGCTGATGA	AGCAGAACAA	CTTTAACGCC	GTGCGCTGTT	CGCATTATCC	2250
GAACCATCCG	CTGTGGTACA	CGCTGTGCGA	CCGCTACGGC	CTGTATGTGG	2300
TGGATGAAGC	CAATATTGAA	ACCCACGGCA	TGGTGCCAAT	GAATCGTCTG	2350
ACCGATGATC	CGCGCTGGCT	ACCGGCGATG	AGCGAACGCG	TAACGCGAAT	2400
GGTGACGCGC	GATCGTAATC	ACCCGAGTGT	GATCATCTGG	TCGCTGGGGA	2450
ATGAATCAGG	CCACGGCGCT	AATCACGACG	CGCTGTATCG	CTGGATCAAA	2500
TCTGTGATC	CTTCCCGCCC	GGTGCAGTAT	GAAGGCGGCG	GAGCCGACAC	2550
CACGGCCACC	GATATTATTT	GCCCGATGTA	CGCGCGCGTG	GATGAAGACC	2600
AGCCCTTCCC	GGCTGTGCCG	AAATGGTCCA	TCAAAAAATG	GCTTTCGCTA	2650
CCTGGAGAGA	CGCGCCCGCT	GATCCTTTGC	GAATACGCCC	ACGCGATGGG	2700
TAACAGTCTT	GGCGGTTTCG	CTAAATACTG	GCAGGCGTTT	CGTCAGTATC	2750
CCCGTTTACA	GGGCGGCTTC	GTCTGGGACT	GGGTGGATCA	GTCGCTGATT	2800
AAATATGATG	AAAACGGCAA	CCCGTGGTCG	GCTTACGGCG	GTGATTTTGG	2850
CGATACGCCG	AACGATCGCC	AGTTCTGTAT	GAACGGTCTG	GTCTTTGCCG	2900

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ACCGCACGCC	GCATCCAGCG	CTGACGGAAG	CAAAACACCA	GCAGCAGTTT	2950
TTCCAGTTCC	GTTTATCCGG	GCAAACCATC	GAAGTGACCA	GCGAATACCT	3000
GTTCCGTCAT	AGCGATAACG	AGCTCCTGCA	CTGGATGGTG	GCGCTGGATG	3050
GTAAGCCGCT	GGCAAGCGGT	GAAGTGCCTC	TGGATGTCGC	TCCACAAGGT	3100
AAACAGTTGA	TTGAACTGCC	TGAACTACCG	CAGCCGGAGA	GCGCCGGGCA	3150
ACTCTGGCTC	ACAGTACGCG	TAGTGCAACC	GAACGCGACC	GCATGGTCAG	3200
AAGCCGGGCA	CATCAGCGCC	TGGCAGCAGT	GGCGTCTGGC	GGAAAACCTC	3250
AGTGTGACGC	TCCCCGCCGC	GTCCCACGCC	ATCCCGCATC	TGACCACCAG	3300
CGAAATGGAT	TTTTGCATCG	AGCTGGGTAA	TAAGCGTTGG	CAATTTAACC	3350
GCCAGTCAGG	CTTTCTTTCA	CAGATGTGGA	TTGGCGATAA	AAAACAACCTG	3400
CTGACGCCGC	TGCGCGATCA	GTTCACCCGT	GCACCGCTGG	ATAACGACAT	3450
TGGCGTAAGT	GAAGCGACCC	GCATTGACCC	TAACGCCTGG	GTCGAACGCT	3500
GGAAGGCGGC	GGGCCATTAC	CAGGCCGAAG	CAGCGTTGTT	GCAGTGCACG	3550
GCAGATACAC	TTGCTGATGC	GGTGCTGATT	ACGACCGCTC	ACGCGTGGCA	3600
GCATCAGGGG	AAAACCTTAT	TTATCAGCCG	GAAAACCTAC	CGGATTGATG	3650
GTAGTGGTCA	AATGGCGATT	ACCGTTGATG	TTGAAGTGGC	GAGCGATACA	3700
CCGCATCCGG	CGCGGATTGG	CCTGAACTGC	CAGCTGGCGC	AGGTAGCAGA	3750
GCGGGTAAAC	TGGCTCGGAT	TAGGGCCGCA	AGAAAACCTAT	CCCGACCGCC	3800
TTACTGCCGC	CTGTTTTGAC	CGCTGGGATC	TGCCATTGTC	AGACATGTAT	3850
ACCCCGTACG	TCTTCCCGAG	CGAAAACGGT	CTGCGCTGCG	GGACGCGCGA	3900
ATTGAATTAT	GGCCACACAC	AGTGGCGCGG	CGACTTCCAG	TTCAACATCA	3950
GCCGCTACAG	TCAACAGCAA	CTGATGGAAA	CCAGCCATCG	CCATCTGCTG	4000
CACGCGGAAG	AAGGCACATG	GCTGAATATC	GACGGTTTCC	ATATGGGGAT	4050
TGGTGGCGAC	GA CTCCTGGA	GCCCGTCAGT	ATCGGCGGAA	TTACAGCTGA	4100
GCGCCGGTCG	CTACCATTAC	CAGTTGGTCT	GGTGTCAAAA	ATAATAATAA	4150
CCGGGCAGGC	CATGTCTGCC	CGTATTTTCG	GTAAGGAAAT	CCATTATGTA	4200

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CTATTTAAAA	AACACAAACT	TTTGGATGTT	CGGTTTATTC	TTTTTCTTTT	4250
ACTTTTTTAT	CATGGGAGCC	TACTTCCCGT	TTTCCCGAT	TTGGCTACAT	4300
GACATCAACC	ATATCAGCAA	AAGTGATACG	GGTATTATTT	TTGCCGCTAT	4350
TTCTCTGTTC	TCGCTATTAT	TCCAACCGCT	GTTTGGTCTG	CTTTCTGACA	4400
AACTCGGCCT	CGACTCTAGG	CGGCCGCGGG	GATCCAGACA	TGATAAGATA	4450
CATTGATGAG	TTTGGACAAA	CCACAACCTAG	AATGCAGTGA	AAAAAATGCT	4500
TTATTTGTGA	AATTTGTGAT	GCTATTGCTT	TATTTGTAAC	CATTATAAGC	4550
TGCAATAAAC	AAGTTAACAA	CAACAATTGC	ATTCATTTTA	TGTTTCAGGT	4600
TCAGGGGGAG	GTGTGGGAGG	TTTTTTCGGA	TCCTCTAGAG	TCGACCTGCA	4650
GGCTGATCAG	TGGAAGGTGC	TGAGGTACGA	TGAGACCCGC	ACCAGGTGCA	4700
GACCCTGCGA	GTGTGGCGGT	AAACATATTA	GGAACCAGCC	TGTGATGCTG	4750
GATGTGACCG	AGGAGCTGAG	GCCCCGATCAC	TTGGTGCTGG	CCTGCACCCG	4800
CGCTGAGTTT	GGCTCTAGCG	ATGAAGATAC	AGATTGAGGT	ACTGAAATGT	4850
GTGGGCGTGG	CTTAAGGGTG	GGAAAGAATA	TATAAGGTGG	GGGTCTTATG	4900
TAGTTTTGTA	TCTGTTTTGC	AGCAGCCGCC	GCCGCCATGA	GCACCAACTC	4950
GTTTGATGGA	AGCATTGTGA	GCTCATATTT	GACAACGCGC	ATGCCCCCAT	5000
GGGCCGGGGT	GCGTCAGAAT	GTGATGGGCT	CCAGCATTGA	TGGTCGCCCC	5050
GTCCTGCCCC	CAAACCTCTAC	TACCTTGACC	TACGAGACCG	TGTCTGGAAC	5100
GCCGTTGGAG	ACTGCAGCCT	CCGCCGCCGC	TTCAGCCGCT	GCAGCCACCG	5150
CCCGCGGGAT	TGTGACTGAC	TTTGCTTTCC	TGAGCCCGCT	TGCAAGCAGT	5200
GCAGCTTCCC	GTTTCATCCGC	CCGCGATGAC	AAGTTGACGG	CTCTTTTGGC	5250
ACAATTGGAT	TCTTTGACCC	GGGAACTTAA	TGTCGTTTCT	CAGCAGCTGT	5300
TGGATCTGCG	CCAGCAGGTT	TCTGCCCTGA	AGGCTTCCTC	CCCTCCCAAT	5350
GCGGTTTAAA	ACATAAATAA	AAAACCAGAC	TCTGTTTGGA	TTTGGATCAA	5400
GCAAGTGTCT	TGCTGTCTTT	ATTTAGGGGT	TTTGCGCGCG	CGGTAGGCCC	5450
GGGACCAGCG	GTCTCGGTCT	TTGAGGGTCC	TGTGTATTTT	TTCCAGGACG	5500

TGGTAAAGGT	GACTCTGGAT	G TTCAGATAC	ATGGGCATAA	GCCCGTCTCT	5550
GGGGTGGAGG	TAGCACC ACT	GCAGAGCTTC	ATGCTGCGGG	GTGGTGT TGT	5600
AGATGATCCA	GTCGTAGCAG	GAGCGCTGGG	CGTGGTGCCT	AAAAATGTCT	5650
TTCAGTAGCA	AGCTGATTGC	CAGGGGCAGG	CCCTTGGTGT	AAGTGTTTAC	5700
AAAGCGGTTA	AGCTGGGATG	GGTGCATACG	TGGGGATATG	AGATGCATCT	5750
TGGACTGTAT	TTTTAGGTTG	GCTATGTTCC	CAGCCATATC	CCTCCGGGGA	5800
TTCATGTTGT	GCAGAACCAC	CAGCACAGTG	TATCCGGTGC	ACTTGGGAAA	5850
TTTGTCATGT	AGCTTAGAAG	GAAATGCGTG	GAAGAACTTG	GAGACGCCCT	5900
TGTGACCTCC	AAGATTTTCC	ATGCATTCGT	CCATAATGAT	GGCAATGGGC	5950
CCACGGGCGG	CGGCCTGGGC	GAAGATATTT	CTGGGATCAC	TAACGTCATA	6000
GTTGTGTTCC	AGGATGAGAT	CGTCATAGGC	CATTTT TACA	AAGCGCGGGC	6050
GGAGGGTGCC	AGACTGCGGT	ATAATGGTTC	CATCCGGCCC	AGGGGCGTAG	6100
TTACCCTCAC	AGATTTGCAT	TTCCCACGCT	TTGAGTTCAG	ATGGGGGGGAT	6150
CATGTCTACC	TGCGGGGCGA	TGAAGAAAAC	GGTTTCCGGG	G TAGGGGAGA	6200
TCAGCTGGGA	AGAAAGCAGG	TTCCTGAGCA	GCTGCGACTT	ACCGCAGCCG	6250
GTGGGCCCCG	AAATCACACC	TATTACCGGG	TGCAACTGGT	AGTTAAGAGA	6300
GCTGCAGCTG	CCGTCATCCC	TGAGCAGGGG	GGCCACTTCG	TTAAGCATGT	6350
CCCTGACTCG	CATGTTTTCC	CTGACCAAAT	CCGCCAGAAG	GCGCTCGCCG	6400
CCCAGCGATA	GCAGTTCTTG	CAAGGAAGCA	AAGTTTTTCA	ACGGTTTGAG	6450
ACCGTCCGCC	G TAGGCATGC	TTTTGAGCGT	TTGACCAAGC	AGTTCCAGGC	6500
GGTCCCACAG	CTCGGTCACC	TGCTCTACGG	CATCTCGATC	CAGCATATCT	6550
CCTCGTTTTCG	CGGGTTGGGG	CGGCTTTCGC	TGTACGGCAG	TAGTCGGTGC	6600
TCGTCCAGAC	GGGCCAGGGT	CATGTCTTTC	CACGGGCGCA	GGGTCCCTCGT	6650
CAGCGTAGTC	TGGGTCACGG	TGAAGGGGTG	CGCTCCGGGC	TGCGCGCTGG	6700
CCAGGGTGCG	CTTGAGGCTG	GTCCTGCTGG	TGCTGAAGCG	CTGCCGGTCT	6750
TCGCCCTGCG	CGTCGGCCAG	G TAGCATTTG	ACCATGGTGT	CATAGTCCAG	6800

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CCCCGCCGCG	GCGTGGCCCT	TGGCGCGCAG	CTTGCCCTTG	GAGGAGGCGC	6850
CGCACGAGGG	GCAGTGCAGA	CTTTTGAGGG	CGTAGAGCTT	GGGCGCGAGA	6900
AATACCGATT	CCGGGGAGTA	GGCATCCGCG	CCGCAGGCCC	CGCAGACGGT	6950
CTCGCATTC	ACGAGCCAGG	TGAGCTCTGG	CCGTTCTGGG	TCAAAAACCA	7000
GGTTTCCCCC	ATGCTTTTTG	ATGCGTTTCT	TACCTCTGGT	TTCCATGAGC	7050
CGGTGTCCAC	GCTCGGTGAC	GAAAAGGCTG	TCCGTGTCCC	CGTATACAGA	7100
CTTGAGAGGC	CTGTCCTCGA	GCGGTGTTCC	GCGGTCCTCC	TCGTATAGAA	7150
ACTCGGACCA	CTCTGAGACA	AAGGCTCGCG	TCCAGGCCAG	CACGAAGGAG	7200
GCTAAGTGGG	AGGGGTAGCG	GTCGTTGTCC	ACTAGGGGGT	CCACTCGCTC	7250
CAGGGTGTGA	AGACACATGT	CGCCCTCTTC	GGCATCAAGG	AAGGTGATTG	7300
GTTTGTAGGT	GTAGGCCACG	TGACCGGGTG	TTCCTGAAGG	GGGGCTATAA	7350
AAGGGGGTGG	GGGCGCGTTC	GTCCTCACTC	TCTTCCGCAT	CGCTGTCTGC	7400
GAGGGCCAGC	TGTTGGGGTG	AGTACTCCCT	CTGAAAAGCG	GGCATGACTT	7450
CTGCGCTAAG	ATTGTCAGTT	TCCAAAAACG	AGGAGGATTT	GATATTACCC	7500
TGGCCCGCGG	TGATGCCTTT	GAGGGTGGCC	GCATCCATCT	GGTCAGAAAA	7550
GACAATCTTT	TTGTTGTCAA	GCTTGGTGGC	AAACGACCCG	TAGAGGGCGT	7600
TGGACAGCAA	CTTGGCGATG	GAGCGCAGGG	TTTGGTTTTT	GTCGCGATCG	7650
GCGCGCTCCT	TGGCCGCGAT	GTTTAGCTGC	ACGTATTCGC	GCGCAACGCA	7700
CCGCCATTCT	GGAAAGACGG	TGGTGCGCTC	GTCGGGCACC	AGGTGCACGC	7750
GCCAACCGCG	GTTGTGCAGG	GTGACAAGGT	CAACGCTGGT	GGCTACCTCT	7800
CCGCGTAGGC	GCTCGTTGGT	CCAGCAGAGG	CGGCCGCCCT	TGCGCGAGCA	7850
GAATGGCGGT	AGGGGGTCTA	GCTGCGTCTC	GTCCGGGGGG	TCTGCGTCCA	7900
CGGTAAAGAC	CCCGGGCAGC	AGGCGCGCGT	CGAAGTAGTC	TATCTTG CAT	7950
CCTTGCAAGT	CTAGCGCCTG	CTGCCATGCG	CGGGCGGCAA	GCGCGCGCTC	8000
GTATGGGTTG	AGTGGGGGAC	CCCATGGCAT	GGGGTGGGTG	AGCGCGGAGG	8050
CGTACATGCC	GCAAATGTCT	TAAACGTAGA	GGGGCTCTCT	GAGTATTCCA	8100

AGATATGTAG	GGTAGCATCT	TCCACCGCGG	ATGCTGGCGC	GCACGTAATC	8150
GTATAGTTCG	TGCGAGGGAG	CGAGGAGGTC	GGGACCGAGG	TTGCTACGGG	8200
CGGGCTGCTC	TGCTCGGAAG	ACTATCTGCC	TGAAGATGGC	ATGTGAGTTG	8250
GATGATATGG	TTGGACGCTG	GAAGACGTTG	AAGCTGGCGT	CTGTGAGACC	8300
TACCGCGTCA	CGCACGAAGG	AGGCGTAGGA	GTCGCGCAGC	TTGTTGACCA	8350
GCTCGGCGGT	GACCTGCACG	TCTAGGGCGC	AGTAGTCCAG	GGTTTCCTTG	8400
ATGATGTCAT	ACTTATCCTG	TCCCTTTTTT	TTCCACAGCT	CGCGGTTGAG	8450
GACAAACTCT	TCGCGGTCTT	TCCAGTACTC	TTGGATCGGA	AACCCGTCGG	8500
CCTCCGAACG	GTAAGAGCCT	AGCATGTAGA	ACTGGTTGAC	GGCCTGGTAG	8550
GCGCAGCATC	CCTTTTCTAC	GGGTAGCGCG	TATGCCTGCG	CGGCCTTCCG	8600
GAGCGAGGTG	TGGGTGAGCG	CAAAGGTGTC	CCTGACCATG	ACTTTGAGGT	8650
ACTGGTATTT	GAAGTCAGTG	TCGTGCGATC	CGCCCTGCTC	CCAGAGCAAA	8700
AAGTCCGTGC	GCTTTTGGGA	ACGCGGATTT	GGCAGGGCGA	AGGTGACATC	8750
GTTGAAGAGT	ATCTTTCCCG	CGCGAGGCAT	AAAGTTGCGT	GTGATGCGGA	8800
AGGGTCCCGG	CACCTCGGAA	CGGTGTGTAA	TTACCTGGGC	GGCGAGCACG	8850
ATCTCGTCAA	AGCCGTTGAT	GTTGTGGCCC	ACAATGTAAA	GTTCCAAGAA	8900
GCGCGGGATG	CCCTTGATGG	AAGGCAATTT	TTTAAGTTCC	TCGTAGGTGA	8950
GCTCTTCAGG	GGAGCTGAGC	CCGTGCTCTG	AAAGGGCCCA	GTCTGCAAGA	9000
TGAGGGTTGG	AAGCGACGAA	TGAGCTCCAC	AGGTCACGGG	CCATTAGCAT	9050
TTGCAGGTGG	TCGCGAAAGG	TCCTAAACTG	GCGACCTATG	GCCATTTTTT	9100
CTGGGGTGAT	GCAGTAGAAG	GTAAGCGGGT	CTTGTTCCCA	GCGGTCCCAT	9150
CCAAGGTTCC	CGGCTAGGTC	TCGCGCGGCA	GTCACTAGAG	GCTCATCTCC	9200
GCCGAAC TTC	ATGACCAGCA	TGAAGGGCAC	GAGCTGCTTC	CCAAAGGCCC	9250
CCATCCAAGT	ATAGGTCTCT	ACATCGTAGG	TGACAAAGAG	ACGCTCGGTG	9300
CGAGGATGCG	AGCCGATCGG	GAAGAACTGG	ATCTCCCGCC	ACCAATTGGA	9350
GGAGTGGCTA	TTGATGTGGT	GAAAGTAGAA	GTCCCTGCGA	CGGGCCGAAC	9400

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ACTCGTGCTG	GCTTTTGTAA	AAACGTGCGC	AGTACTGGCA	GCGGTGCACG	9450
GGCTGTACAT	CCTGCACGAG	GTTGACCTGA	CGACCGCGCA	CAAGGAAGCA	9500
GAGTGGAAT	TTGAGCCCCT	CGCCTGGCGG	GTTTGGCTGG	TGGTCTTCTA	9550
CTTCGGCTGC	TTGTCCTTGA	CCGTCTGGCT	GCTCGAGGGG	AGTTACGGTG	9600
GATCGGACCA	CCACGCCGCG	CGAGCCCAA	GTCCAGATGT	CCGCGCGCGG	9650
CGGTCGGAGC	TTGATGACAA	CATCGCGCAG	ATGGGAGCTG	TCCATGGTCT	9700
GGAGCTCCCG	CGGCGTCAGG	TCAGGCGGGA	GCTCCTGCAG	GTTTACCTCG	9750
CATAGACGGG	TCAGGGCGCG	GGCTAGATCC	AGGTGATACC	TAATTTCCAG	9800
GGGCTGGTTG	GTGGCGGCGT	CGATGGCTTG	CAAGAGGCCG	CATCCCCGCG	9850
GCGCGACTAC	GGTACGCGC	GGCGGGCGGT	GGGCCGCGGG	GGTGTCTTTC	9900
GATGATGCAT	CTAAAAGCGG	TGACGCGGGC	GAGCCCCCGG	AGGTAGGGGG	9950
GGCTCCGGAC	CCGCCGGGAG	AGGGGGCAGG	GGCACGTCGG	CGCCGCGCGC	10000
GGGCAGGAGC	TGGTGCTGCG	CGCGTAGGTT	GCTGGCGAAC	GCGACGACGC	10050
GGCGGTTGAT	CTCCTGAATC	TGGCGCCTCT	GCGTGAAGAC	GACGGGGCCG	10100
GTGAGCTTGA	GCCTGAAAGA	GAGTTCGACA	GAATCAATTT	CGGTGTCGTT	10150
GACGGCGGGC	TGGCGCAAAA	TCTCCTGCAC	GTCTCCTGAG	TTGTCTTGAT	10200
AGGCGATCTC	GGCCATGAAC	TGCTCGATCT	CTTCCTCCTG	GAGATCTCCG	10250
CGTCCGGCTC	GCTCCACGGT	GGCGGCGAGG	TCGTTGGAAA	TGCGGGCCAT	10300
GAGCTGCGAG	AAGGCGTTGA	GGCCTCCCTC	GTTCCAGACG	CGGCTGTAGA	10350
CCACGCCCCC	TTCGGCATCG	CGGGCGCGCA	TGACCACCTG	CGCGAGATTG	10400
AGCTCCACGT	GCCGGGCGAA	GACGGCGTAG	TTTCGCAGGC	GCTGAAAGAG	10450
GTAGTTGAGG	GTGGTGGCGG	TGTGTTCTGC	CACGAAGAAG	TACATAACCC	10500
AGCGTCGCAA	CGTGGATTTC	TTGATATCCC	CCAAGGCCTC	AAGGCGCTCC	10550
ATGGCCTCGT	AGAAGTCCAC	GGCGAAGTTG	AAAACTGGG	AGTTGCGCGC	10600
CGACACGGTT	AACTCCTCCT	CCAGAAGACG	GATGAGCTCG	GCGACAGTGT	10650
CGCGCACCTC	GCGCTCAAAG	GCTACAGGGG	CCTCTTCTTC	TTCTTCAATC	10700

TCCTCTTCCA	TAAGGGCCTC	CCCTTCTTCT	TCTTCTGGCG	GCGGTGGGGG	10750
AGGGGGGACA	CGGCGGCGAC	GACGGCGCAC	CGGGAGGCGG	TCGACAAAGC	10800
GCTCGATCAT	CTCCCCGCGG	CGACGGCGCA	TGGTCTCGGT	GACGGCGCGG	10850
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8509 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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TGCGCCCATC	TACACCAACG	TAACCTATCC	CATTACGGTC	AATCCGCCGT	1500	
TTGTTCCAC	GGAGAATCCG	ACGGGTGTT	ACTCGCTCAC	ATTTAATGTT	1550	
GATGAAAGCT	GGCTACAGGA	AGGCCAGACG	CGAATTATTT	TTGATGGCGT	1600	
TAACTCGGCG	TTTCATCTGT	GGTGCAACGG	GCGCTGGGTC	GGTTACGGCC	1650	
AGGACAGTCG	TTTGCCGTCT	GAATTTGACC	TGAGCGCATT	TTTACGCGCC	1700	
GGAGAAAACC	GCCTCGCGGT	GATGGTGCTG	CGTTGGAGTG	ACGGCAGTTA	1750	
TCTGGAAGAT	CAGGATATGT	GGCGGATGAG	CGGCATTTTC	CGTGACGTCT	1800	
CGTTGCTGCA	TAAACCGACT	ACACAAATCA	GCGATTTCCA	TGTTGCCACT	1850	
CGCTTTAATG	ATGATTTCAG	CCGCGCTGTA	CTGGAGGCTG	AAGTTCAGAT	1900	
GTGCGGCGAG	TTGCGTGA	ACCTACGGGT	AACAGTTTCT	TTATGGCAGG	1950	
GTGAAACGCA	GGTCGCCAGC	GGCACC	GCGC	CTTTCGGCGG	TGAAATTATC	2000
GATGAGCGTG	GTGGTTATGC	CGATCGCGTC	ACACTACGTC	TGAACGT	CGA	2050
AAACCCGAAA	CTGTGGAGCG	CCGAAATCCC	GAATCTCTAT	CGTGCGGTGG		2100
TTGAACTGCA	CACCGCCGAC	GGCACGCTGA	TTGAAGCAGA	AGCCTGCGAT		2150
GTCGGTTTCC	GCGAGGTGCG	GATTGAAAAT	GGTCTGCTGC	TGCTGAACGG		2200

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CAAGCCGTTG	CTGATTCGAG	GCGTTAACCG	TCACGAGCAT	CATCCTCTGC	2250
ATGGTCAGGT	CATGGATGAG	CAGACGATGG	TGCAGGATAT	CCTGCTGATG	2300
AAGCAGAACA	ACTTTAACGC	CGTGCGCTGT	TCGCATTATC	CGAACCATCC	2350
GCTGTGGTAC	ACGCTGTGCG	ACCGCTACGG	CCTGTATGTG	GTGGATGAAG	2400
CCAATATTGA	AACCCACGGC	ATGGTGCCAA	TGAATCGTCT	GACCGATGAT	2450
CCGCGCTGGC	TACCGGCGAT	GAGCGAACGC	GTAACGCGAA	TGGTGCAGCG	2500
CGATCGTAAT	CACCCGAGTG	TGATCATCTG	GTCGCTGGGG	AATGAATCAG	2550
GCCACGGCGC	TAATCACGAC	GCGCTGTATC	GCTGGATCAA	ATCTGTCGAT	2600
CCTTCCCGCC	CGGTGCAGTA	TGAAGGCGGC	GGAGCCGACA	CCACGGCCAC	2650
CGATATTATT	TGCCCGATGT	ACGCGCGCGT	GGATGAAGAC	CAGCCCTTCC	2700
CGGCTGTGCC	GAAATGGTCC	ATCAAAAAAT	GGCTTTCGCT	ACCTGGAGAG	2750
ACGCGCCCGC	TGATCCTTTG	CGAATACGCC	CACGCGATGG	GTAACAGTCT	2800
TGGCGGTTTC	GCTAAATACT	GGCAGGCGTT	TCGTCAGTAT	CCCCGTTTAC	2850
AGGGCGGCTT	CGTCTGGGAC	TGGGTGGATC	AGTCGCTGAT	TAAATATGAT	2900
GAAAACGGCA	ACCCGTGGTC	GGCTTACGGC	GGTGATTTTG	GCGATACGCC	2950
GAACGATCGC	CAGTTCTGTA	TGAACGGTCT	GGTCTTTGCC	GACCGCACGC	3000
CGCATCCAGC	GCTGACGGAA	GCAAAACACC	AGCAGCAGTT	TTTCCAGTTC	3050
CGTTTATCCG	GGCAAACCAT	CGAAGTGACC	AGCGAATACC	TGTTCCGTCA	3100
TAGCGATAAC	GAGCTCCTGC	ACTGGATGGT	GGCGCTGGAT	GGTAAGCCGC	3150
TGGCAAGCGG	TGAAGTGCCT	CTGGATGTCG	CTCCACAAGG	TAAACAGTTG	3200
ATTGAACTGC	CTGAACTACC	GCAGCCGGAG	AGCGCCGGGC	AACTCTGGCT	3250
CACAGTACGC	GTAGTGCAAC	CGAACGCGAC	CGCATGGTCA	GAAGCCGGGC	3300
ACATCAGCGC	CTGGCAGCAG	TGGCGTCTGG	CGGAAAACCT	CAGTGTGACG	3350
CTCCCCGCCG	CGTCCCACGC	CATCCCGCAT	CTGACCACCA	GCGAAATGGA	3400
TTTTTGCATC	GAGCTGGGTA	ATAAGCGTTG	GCAATTTAAC	CGCCAGTCAG	3450
GCTTTCTTTC	ACAGATGTGG	ATTGGCGATA	AAAAACAAC	GCTGACGCCG	3500

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CTGCGCGATC	AGTTCACCCG	TGCACCGCTG	GATAACGACA	TTGGCGTAAG	3550
TGAAGCGACC	CGCATTGACC	CTAACGCCTG	GGTCGAACGC	TGGAAGGCGG	3600
CGGGCCATTA	CCAGGCCGAA	GCAGCGTTGT	TGCAGTGCAC	GGCAGATACA	3650
CTTGCTGATG	CGGTGCTGAT	TACGACCGCT	CACGCGTGGC	AGCATCAGGG	3700
GAAAACCTTA	TTTATCAGCC	GGAAAACCTA	CCGGATTGAT	GGTAGTGGTC	3750
AAATGGCGAT	TACCGTTGAT	GTTGAAGTGG	CGAGCGATAC	ACCGCATCCG	3800
GCGCGGATTG	GCCTGAACTG	CCAGCTGGCG	CAGGTAGCAG	AGCGGGTAAA	3850
CTGGCTCGGA	TTAGGGCCGC	AAGAAAACCTA	TCCCGACCGC	CTTACTGCCG	3900
CCTGTTTTGA	CCGCTGGGAT	CTGCCATTGT	CAGACATGTA	TACCCCGTAC	3950
GTCTTCCCGA	GCGAAAACGG	TCTGCGCTGC	GGGACGCGCG	AATTGAATTA	4000
TGGCCACAC	CAGTGGCGCG	GCGACTTCCA	GTTCAACATC	AGCCGCTACA	4050
GTCAACAGCA	ACTGATGGAA	ACCAGCCATC	GCCATCTGCT	GCACGCGGAA	4100
GAAGGCACAT	GGCTGAATAT	CGACGGTTTC	CATATGGGGA	TTGGTGGCGA	4150
CGACTCCTGG	AGCCCGTCAG	TATCGGCGGA	ATTACAGCTG	AGCGCCGGTC	4200
GCTACCATTA	CCAGTTGGTC	TGGTGTCAAA	AATAATAATA	ACCGGGCAGG	4250
CCATGTCTGC	CCGTATTTTCG	CGTAAGGAAA	TCCATTATGT	ACTATTTAAA	4300
AAACACAAAC	TTTTGGATGT	TCGGTTTATT	CTTTTTCTTT	TACTTTTTTA	4350
TCATGGGAGC	CTACTTCCCG	TTTTTCCCGA	TTTGGCTACA	TGACATCAAC	4400
CATATCAGCA	AAAGTGATAC	GGGTATTATT	TTTGCCGCTA	TTTCTCTGTT	4450
CTCGCTATTA	TTCCAACCGC	TGTTTGGTCT	GCTTTCTGAC	AAACTCGGCC	4500
TCGACTCTAG	GCGGCCGCGG	GGATCCAGAC	ATGATAAGAT	ACATTGATGA	4550
GTTTGGACAA	ACCACAATA	GAATGCAGTG	AAAAAAATGC	TTTATTTGTG	4600
AAATTTGTGA	TGCTATTGCT	TTATTTGTAA	CCATTATAAG	CTGCAATAAA	4650
CAAGTTAACA	ACAACAATTG	CATTCATTTT	ATGTTTCAGG	TTCAGGGGGA	4700
GGTGTGGGAG	GTTTTTTTCGG	ATCCTCTAGA	GTCGACCTGC	AGGGGCTAGA	4750
ATGGCTACGT	AGATAAGTAG	CATGGCGGGT	TAATCATTA	CTACAAGGAA	4800

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CCCCTAGTGA	TGGAGTTGGC	CACTCCCTCT	CTGCGCGCTC	GCTCGCTCAC	4850
TGAGGCCGGG	CGACCAAAGG	TCGCCCCGACG	CCCGGGCTTT	GGCCGGGCGG	4900
CCTCAGTGAG	CGAGCGAGCG	CGCAGCTGGC	GTAATAGCGA	AGAGGCCCGC	4950
ACCGATCGCC	CTTCCCAACA	GTTGCGCAGC	CTGAATGGCG	AATGGAATTC	5000
CAGACGATTG	AGCGTCAAAA	TGTAGGTATT	TCCATGAGCG	TTTTTCCTGT	5050
TGCAATGGCT	GGCGGTAATA	TTGTTCTGGA	TATTACCAGC	AAGCCCGATA	5100
GTTTGAGTTC	TTCTACTCAG	GCAAGTGATG	TTATTACTAA	TCAAAGAAGT	5150
ATTGCGACAA	CGGTTAATTT	GCGTGATGGA	CAGACTCTTT	TACTCGGTGG	5200
CCTCACTGAT	TATAAAAACA	CTTCTCAGGA	TTCTGGCGTA	CCGTTCCCTGT	5250
CTAAAATCCC	TTTAATCGGC	CTCCTGTTTA	GCTCCCGCTC	TGATTCTAAC	5300
GAGGAAAGCA	CGTTATACGT	GCTCGTCAAA	GCAACCATAG	TACGCGCCCT	5350
GTAGCGGCGC	ATTAAGCGCG	GCGGGTGTGG	TGGTTACGCG	CAGCGTGACC	5400
GCTACACTTG	CCAGCGCCCT	AGCGCCCGCT	CCTTTCGCTT	TCTTCCCTTC	5450
CTTTCTCGCC	ACGTTCTGCCG	GCTTTCCCCG	TCAAGCTCTA	AATCGGGGGC	5500
TCCCTTTAGG	GTTCCGATTT	AGTGCTTTAC	GGCACCTCGA	CCCCAAAAA	5550
CTTGATTAGG	GTGATGGTTC	ACGTAGTGGG	CCATCGCCCT	GATAGACGGT	5600
TTTTCGCCCT	TTGACGTTGG	AGTCCACGTT	CTTTAATAGT	GGACTCTTGT	5650
TCCAAACTGG	AACAACACTC	AACCCTATCT	CGGTCTATTC	TTTTGATTTA	5700
TAAGGGATTT	TGCCGATTTT	GGCCTATTGG	TTAAAAAATG	AGCTGATTTA	5750
ACAAAAATTT	AACGCGAATT	TTAACAAAAT	ATTAACGTTT	ACAATTTAAA	5800
TATTTGCTTA	TACAATCTTC	CTGTTTTTGG	GGCTTTTCTG	ATTATCAACC	5850
GGGGTACATA	TGATTGACAT	GCTAGTTTTA	CGATTACCGT	TCATCGATTTC	5900
TCTTGTTTGC	TCCAGACTCT	CAGGCAATGA	CCTGATAGCC	TTTGTAGAGA	5950
CCTCTCAAAA	ATAGCTACCC	TCTCCGGCAT	GAATTTATCA	GCTAGAACGG	6000
TTGAATATCA	TATTGATGGT	GATTTGACTG	TCTCCGGCCT	TTCTCACCCG	6050
TTTGAATCTT	TACCTACACA	TTACTCAGGC	ATTGCATTTA	AAATATATGA	6100

GGGTTCTAAA	AATTTTATC	CTTGCGTTGA	AATAAAGGCT	TCTCCCGCAA	6150
AAGTATTACA	GGGTCATAAT	GTTTTTGGTA	CAACCGATTT	AGCTTTATGC	6200
TCTGAGGCTT	TATTGCTTAA	TTTTGCTAAT	TCTTTGCCTT	GCCTGTATGA	6250
TTTATTGGAT	GTTGGAATTC	CTGATGCGGT	ATTTTCTCCT	TACGCATCTG	6300
TGCGGTATTT	CACACCGCAT	ATGGTGCACT	CTCAGTACAA	TCTGCTCTGA	6350
TGCCGCATAG	TTAAGCCAGC	CCCACACCC	GCCAACACCC	GCTGACGCGC	6400
CCTGACGGGC	TTGTCTGCTC	CCGGCATCCG	CTTACAGACA	AGCTGTGACC	6450
GTCTCCGGGA	GCTGCATGTG	TCAGAGGTTT	TCACCGTCAT	CACCGAAACG	6500
CGCGAGACGA	AAGGGCCTCG	TGATACGCCT	ATTTTATAG	GTTAATGTCA	6550
TGATAATAAT	GGTTTCTTAG	ACGTCAGGTG	GCACTTTTCG	GGGAAATGTG	6600
CGCGGAACCC	CTATTTGTTT	ATTTTCTAA	ATACATTCAA	ATATGTATCC	6650
GCTCATGAGA	CAATAACCCT	GATAAATGCT	TCAATAATAT	TGAAAAAGGA	6700
AGAGTATGAG	TATTCAACAT	TTCCGTGTCG	CCCTTATTCC	CTTTTTTGCG	6750
GCATTTTGCC	TTCTGTGTTT	TGCTCACCCA	GAAACGCTGG	TGAAAGTAAA	6800
AGATGCTGAA	GATCAGTTGG	GTGCACGAGT	GGGTTACATC	GAAGTGGATC	6850
TCAACAGCGG	TAAGATCCTT	GAGAGTTTTT	GCCCCGAAGA	ACGTTTTCCA	6900
ATGATGAGCA	CTTTTAAAGT	TCTGCTATGT	GGCGCGGTAT	TATCCCGTAT	6950
TGACGCCGGG	CAAGAGCAAC	TCGGTCGCCG	CATACACTAT	TCTCAGAATG	7000
ACTTGGTTGA	GTAATCACCA	GTCACAGAAA	AGCATCTTAC	GGATGGCATG	7050
ACAGTAAGAG	AATTATGCAG	TGCTGCCATA	ACCATGAGTG	ATAACACTGC	7100
GGCCAACTTA	CTTCTGACAA	CGATCGGAGG	ACCGAAGGAG	CTAACCGCTT	7150
TTTTGCACAA	CATGGGGGAT	CATGTAAGTC	GCCTTGATCG	TTGGGAACCG	7200
GAGCTGAATG	AAGCCATACC	AAACGACGAG	CGTGACACCA	CGATGCCTGT	7250
AGCAATGGCA	ACAACGTTGC	GCAAACTATT	AACTGGCGAA	CTACTTACTC	7300
TAGCTTCCCG	GCAACAATTA	ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	7350
GGACCACTTC	TGCGCTCGGC	CCTTCCGGCT	GGCTGGTTTA	TTGCTGATAA	7400

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ATCTGGAGCC	GGTGAGCGTG	GGTCTCGCGG	TATCATTGCA	GCACTGGGGC	7450
CAGATGGTAA	CCCCTCCCGT	ATCGTAGTTA	TCTACACGAC	GGGGAGTCAG	7500
GCAACTATGG	ATGAACGAAA	TAGACAGATC	GCTGAGATAG	GTGCCTCACT	7550
GATTAAGCAT	TGGTAACTGT	CAGACCAAGT	TTACTCATAT	ATACTTTAGA	7600
TTGATTTAAA	ACTTCATTTT	TAATTTAAAA	GGATCTAGGT	GAAGATCCTT	7650
TTTGATAATC	TCATGACCAA	AATCCCTTAA	CGTGAGTTTT	CGTTCCACTG	7700
AGCGTCAGAC	CCCGTAGAAA	AGATCAAAGG	ATCTTCTTGA	GATCCTTTTT	7750
TTCTGCGCGT	AATCTGCTGC	TTGCAAACAA	AAAAACCACC	GCTACCAGCG	7800
GTGGTTTGTT	TGCCGGATCA	AGAGCTACCA	ACTCTTTTTC	CGAAGGTAAC	7850
TGGCTTCAGC	AGAGCGCAGA	TACCAAATAC	TGTCCTTCTA	GTGTAGCCGT	7900
AGTTAGGCCA	CCACTTCAAG	AACTCTGTAG	CACCGCCTAC	ATACCTCGCT	7950
CTGCTAATCC	TGTTACCAGT	GGCTGCTGCC	AGTGGCGATA	AGTCGTGTCT	8000
TACCGGGTTG	GA CTCAAGAC	GATAGTTACC	GGATAAGGCG	CAGCGGTCGG	8050
GCTGAACGGG	GGGTTCGTGC	ACACAGCCCA	GCTTGGAGCG	AACGACCTAC	8100
ACCGAACTGA	GATACCTACA	GCGTGAGCTA	TGAGAAAGCG	CCACGCTTCC	8150
CGAAGGGAGA	AAGGCGGACA	GGTATCCGGT	AAGCGGCAGG	GTCGGAACAG	8200
GAGAGCGCAC	GAGGGAGCTT	CCAGGGGGAA	ACGCCTGGTA	TCTTTATAGT	8250
CCTGTCGGGT	TTCGCCACCT	CTGACTTGAG	CGTCGATTTT	TGTGATGCTC	8300
GTCAGGGGGG	CGGAGCCTAT	GGAAAAACGC	CAGCAACGCG	GCCTTTTTAC	8350
GGTTCCTGGC	CTTTTGCTGG	CCTTTTGCTC	ACATGTTCTT	TCCTGCGTTA	8400
TCCCCTGATT	CTGTGGATAA	CCGTATTACC	GCCTTTGAGT	GAGCTGATAC	8450
CGCTCGCCGC	AGCCGAACGA	CCGAGCGCAG	CGAGTCAGTG	AGCGAGGAAG	8500
CGGAAGAGC					8509

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8299 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCCAATACG CAAACCGCCT CTCCCCGCGC GTTGGCCGAT TCATTAATGC	50
AGCTGCGCGC TCGCTCGCTC ACTGAGGCCG CCCGGGCAAA GCCCGGGCGT	100
CGGGCGACCT TTGGTCGCCC GGCCTCAGTG AGCGAGCGAG CGCGCAGAGA	150
GGGAGTGGCC AACTCCATCA CTAGGGGTTC CTTGTAGTTA ATGATTAACC	200
CGCCATGCTA CTTATCTACA TCATCGATGA ATTCGAGCTT GCATGCCTGC	250
AGGTCGTTAC ATAACCTACG GTAAATGGCC CGCCTGGCTG ACCGCCCAAC	300
GACCCCCGCC CATTGACGTC AATAATGACG TATGTTCCCA TAGTAACGCC	350
AATAGGGACT TTCCATTGAC GTCAATGGGT GGAGTATTTA CGGTAAACTG	400
CCCACCTGGC AGTACATCAA GTGTATCATA TGCCAAGTAC GCCCCCTATT	450
GACGTCAATG ACGGTAAATG GCCCGCCTGG CATTATGCCC AGTACATGAC	500
CTTATGGGAC TTTCCTACTT GGCAGTACAT CTACGTATTA GTCATCGCTA	550
TTACCATGGT GATGCGGTTT TGGCAGTACA TCAATGGGCG TGGATAGCGG	600
TTTGACTCAC GGGGATTTCC AAGTCTCCAC CCCATTGACG TCAATGGGAG	650
TTTGTTTTGG CACCAAAATC AACGGGACTT TCCAAAATGT CGTAACAACT	700
CCGCCCCATT GACGCAAATG GGCGGTAGGC GTGTACGGTG GGAGGTCTAT	750
ATAAGCAGAG CTCGTTTAGT GAACCGTCAG ATCGCCTGGA GACGCCATCC	800
ACGCTGTTTT GACCTCCATA GAAGACACCG GGACCGATCC AGCCTCCGGA	850
CTCTAGAGGA TCCGGTACTC GACCCGAGCT CGGATCCACT AGTAACGGCC	900
GCCAGTGTGC TGGAAATTCTG CACTCCAGGC TGCCCGGGTT TGCATGCTGC	950
TGCTGCTGCT GCTGCTGGGC CTGAGGCTAC AGCTCTCCCT GGGCATCATC	1000

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CTAGTTGAGG	AGGAGAACCC	GGACTTCTGG	AACCGCGAGG	CAGCCGAGGC	1050
CCTGGGTGCC	GCCAAGAAGC	TGCAGCCTGC	ACAGACAGCC	GCCAAGAACC	1100
TCATCATCTT	CCTGGGCGAT	GGGATGGGGG	TGTCTACGGT	GACAGCTGCC	1150
AGGATCCTAA	AAGGGCAGAA	GAAGGACAAA	CTGGGGCCTG	AGATACCCCT	1200
GGCCATGGAC	CGCTTCCCAT	ATGTGGCTCT	GTCCAAGACA	TACAATGTAG	1250
ACAAACATGT	GCCAGACAGT	GGAGCCACAG	CCACGGCCTA	CCTGTGCGGG	1300
GTCAAGGGCA	ACTTCCAGAC	CATTGGCTTG	AGTGCAGCCG	CCCGCTTTAA	1350
CCAGTGCAAC	ACGACACGCG	GCAACGAGGT	CATCTCCGTG	ATGAATCGGG	1400
CCAAGAAAGC	AGGGAAGTCA	GTGGGAGTGG	TAACCACCAC	ACGAGTGCAG	1450
CACGCCTCGC	CAGCCGGCAC	CTACGCCCAC	ACGGTGAACC	GCAACTGGTA	1500
CTCGGACGCC	GACGTGCCTG	CCTCGGCCCG	CCAGGAGGGG	TGCCAGGACA	1550
TCGCTACGCA	GCTCATCTCC	AACATGGACA	TTGATGTGAT	CCTAGGTGGA	1600
GGCCGAAAGT	ACATGTTTCG	CATGGGAACC	CCAGACCCTG	AGTACCCAGA	1650
TGACTACAGC	CAAGGTGGGA	CCAGGCTGGA	CGGGAAGAAT	CTGGTGCAGG	1700
AATGGCTCGG	CGAACGCCAG	GGTGCCCGGT	ACGTGTGGAA	CCGCACTGAG	1750
CTCATGCAGG	CTTCCCTGGA	CCCGTCTGTG	ACCCATCTCA	TGGGTCTCTT	1800
TGAGCCTGGA	GACATGAAAT	ACGAGATCCA	CCGAGACTCC	AACTGGACC	1850
CCTCCCTGAT	GGAGATGACA	GAGGCTGCCC	TGCGCCTGCT	GAGCAGACAC	1900
CCCCGCGGCT	TCTTCCTCTT	CGTGGAGGGT	GGTCGCATCG	ACCATGGTCA	1950
TCATGAAAGC	AGGGCTTACC	GGGCACTGAC	TGAGACGATC	ATGTTTCGACG	2000
ACGCCATTGA	GAGGGCGGGC	CAGCTCACCA	GCGAGGAGGA	CACGCTGAGC	2050
CTCGTCACTG	CCGACCACTC	CCACGTCTTC	TCCTTCGGAG	GCTACCCCTT	2100
GCGAGGGAGC	TCCTTCATCG	GGCTGGCCGC	TGGCAAGGCC	CGGGACAGGA	2150
AGGCCTACAC	GGTCCTCCTA	TACGGAAACG	GTCCAGGCTA	TGTGCTCAAG	2200
GACGGCGCCC	GGCCGGATGT	TACCGAGAGC	GAGAGCGGGA	GCCCCGAGTA	2250
TCGGCAGCAG	TCAGCAGTGC	CCCTGGACGA	AGAGACCCAC	GCAGGCGAGG	2300

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ACGTGGCGGT	GTTCGCGCGC	GGCCCGCAGG	CGCACCTGGT	TCACGGCGTG	2350
CAGGAGCAGA	CCTTCATAGC	GCACGTCATG	GCCTTCGCCG	CCTGCCTGGA	2400
GCCCTACACC	GCCTGCGACC	TGGCGCCCCC	CGCCGGCACC	ACCGACGCCG	2450
CGCACCCGGG	GCGGTCCGTG	GTCCCCGCGT	TGCTTCCTCT	GCTGGCCGGG	2500
ACCCTGCTGC	TGCTGGAGAC	GGCCACTGCT	CCCTGAGTGT	CCCGTCCCTG	2550
GGGCTCCTGC	TTCCCCATCC	CGGAGTTCTC	CTGCTCCCCA	CCTCCTGTCTG	2600
TCCTGCCTGG	CCTCCAGCCC	GAGTCGTCAT	CCCCGGAGTC	CCTATACAGA	2650
GGTCCTGCCA	TGGAACCTTC	CCCTCCCCGT	GCGCTCTGGG	GA CTGAGCCC	2700
ATGACACCAA	ACCTGCCCCCT	TGGCTGCTCT	CGGACTCCCT	ACCCCAACCC	2750
CAGGGACTGC	AGGTTGTGCC	CTGTGGCTGC	CTGCACCCCA	GGAAAGGAGG	2800
GGGCTCAGGC	CATCCAGCCA	CCACCTACAG	CCCAGTGGGG	TCGAGACAGA	2850
TGGTCAGTCT	GGAGGATGAC	GTGGCGTGAA	GCTGGCCGCG	GGGATCCAGA	2900
CATGATAAGA	TACATTGATG	AGTTTGGACA	AACCACAACT	AGAATGCAGT	2950
GAAAAAATG	CTTTATTTGT	GAAATTTGTG	ATGCTATTGC	TTTATTTGTA	3000
ACCATTATAA	GCTGCAATAA	ACAAGTTAAC	AACAACAATT	GCATTCATTT	3050
TATGTTTCAG	GTTCAGGGGG	AGGTGTGGGA	GGTTTTTTTCG	GATCCTCTAG	3100
AGTCGACTCT	AGANNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3150
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3200
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3250
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3300
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3350
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNGGATCCC	3400
CATGACTACG	TCCGGCGTTC	CATTTGGCAT	GACACTACGA	CCAACACGAT	3450
CTCGGTTGTC	TCGGCGCACT	CCGTACAGTA	GGGATCGTCT	ACCTCCTTTT	3500
GAGACAGAAA	CCCGCGCTAC	CATACTGGAG	GATCATCCGC	TGCTGCCCCGA	3550
ATGTAACACT	TTGACAATGC	ACAACGTGAG	TTACGTGCGA	GGTCTTCCCT	3600

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GCAGTGTGGG	ATTTACGCTG	ATTCAGGAAT	GGGTTGTTCC	CTGGGATATG	3650
GTTCTAACGC	GGGAGGAGCT	TGTAATCCTG	AGGAAGTGTA	TGCACGTGTG	3700
CCTGTGTTGT	GCCAACATTG	ATATCATGAC	GAGCATGATG	ATCCATGGTT	3750
ACGAGTCCTG	GGCTCTCCAC	TGTCATTGTT	CCAGTCCCGG	TTCCCTGCAG	3800
TGTATAGCCG	GCGGGCAGGT	TTTGGCCAGC	TGGTTTAGGA	TGGTGGTGGA	3850
TGGCGCCATG	TTTAATCAGA	GGTTTATATG	GTACCGGGAG	GTGGTGAATT	3900
ACAACATGCC	AAAAGAGGTA	ATGTTTATGT	CCAGCGTGTT	TATGAGGGGT	3950
CGCCACTTAA	TCTACCTGCG	CTTGTGGTAT	GATGGCCACG	TGGGTTCTGT	4000
GGTCCCCGCC	ATGAGCTTTG	GATACAGCGC	CTTGCACTGT	GGGATTTTGA	4050
ACAATATTGT	GGTGCTGTGC	TGCAGTTACT	GTGCTGATTT	AAGTGAGATC	4100
AGGGTGCGCT	GCTGTGCCCC	GAGGACAAGG	CGCCTTATGC	TGCGGGCGGT	4150
GCGAATCATC	GCTGAGGAGA	CCACTGCCAT	GTTGTATTCC	TGCAGGACGG	4200
AGCGGCGGCG	GCAGCAGTTT	ATTGCGCGCG	TGCTGCAGCA	CCACCGCCCT	4250
ATCCTGATGC	ACGATTATGA	CTCTACCCCC	ATGTAGGGAT	CCCCATCACT	4300
AGTGCGGCCG	CGGGGATCCA	GACATGATAA	GATACATTGA	TGAGTTTGGA	4350
CAAACCACAA	CTAGAATGCA	GTGAAAAAAA	TGCTTTATTT	GTGAAATTTG	4400
TGATGCTATT	GCTTTATTTG	TAACCATTAT	AAGCTGCAAT	AAACAAGTTA	4450
ACAACAACAA	TTGCATTTCAT	TTTATGTTTC	AGGTTCAAGG	GGAGGTGTGG	4500
GAGGTTTTTT	CGGATCCTCT	AGAGTCGACC	TGCAGGCATG	CAAGCTGTAG	4550
ATAAGTAGCA	TGGCGGGTTA	ATCATTA ACT	ACAAGGAACC	CCTAGTGATG	4600
GAGTTGGCCA	CTCCCTCTCT	GCGCGCTCGC	TCGCTCACTG	AGGCCGGGCG	4650
ACCAAAGGTC	GCCCCACGCC	CGGGCTTTGC	CCGGGCGGCC	TCAGTGAGCG	4700
AGCGAGCGCG	CAGCTGGCGT	AATAGCGAAG	AGGCCCGCAC	CGATCGCCCT	4750
TCCCAACAGT	TGCGCAGCCT	GAATGGCGAA	TGGAANTTCC	AGACGATTGA	4800
GCGTCAAAAT	GTAGGTATTT	CCATGAGCGT	TTTTCTGTGTT	GCAATGGCTG	4850
GCGGTAATAT	TGTTCTGGAT	ATTACCAGCA	AGGCCGATAG	TTTGAGTTCT	4900

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TCTACTCAGG	CAAGTGATGT	TATTACTAAT	CAAAGAAGTA	TTGCGACAAC	4950
GGTTAATTTG	CGTGATGGAC	AGACTCTTTT	ACTCGGTGGC	CTCACTGATT	5000
ATAAAAACAC	TTCTCAGGAT	TCTGGCGTAC	CGTTCCTGTC	TAAAATCCCT	5050
TTAATCGGCC	TCCTGTTTAG	CTCCCGCTCT	GATTCTAACG	AGGAAAGCAC	5100
GTTATACGTG	CTCGTCAAAG	CAACCATAGT	ACGCGCCCTG	TAGCGGCGCA	5150
TTAAGCGCGG	CGGGTGTGGT	GGTTACGCGC	AGCGTGACCG	CTACACTTGC	5200
CAGCGCCCTA	GCGCCCGCTC	CTTTCGCTTT	CTTCCCTTCC	TTTCTCGCCA	5250
CGTTCGCCGG	CTTTCCTCGT	CAAGCTCTAA	ATCGGGGGCT	CCCTTTAGGG	5300
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AGCCGAACGA	CCGAGCGCAG	CGAGTCAGTG	AGCGAGGAAG	CGGAAGAGC	8299

WHAT IS CLAIMED IS:

1. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell *ex vivo* comprising the steps of providing a recombinant adeno-associated virus comprising: (a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing a selected gene into a target cell in the absence of cell division; and (b) a selected gene operatively linked to regulatory sequences directing its expression, said gene flanked by the DNA of (a) and capable of expression in the target cell; infecting said target cells with said recombinant adeno-associated virus; contacting said infected cells with an agent which facilitates the conversion of said ss recombinant virus to its double stranded form, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus into said target cell.
2. The method according to claim 1 wherein said agent is a helper virus comprising a selected gene encoding a polypeptide which can enhance the conversion of said single-stranded recombinant virus to double-stranded recombinant virus or functional fragment thereof, said helper virus capable of transducing said selected gene into the target cell in the absence of cell division.
3. The method according to claim 2 wherein said contacting step comprises co-infecting said target cell with said helper virus.
4. The method according to claim 3 wherein said helper virus is an adenovirus.

5. The method according to claim 2 wherein said selected gene is the adenovirus E4 gene or a functional fragment thereof.

6. The method according to claim 2 wherein said adenovirus contains two selected genes or functional fragments thereof, said genes being the adenovirus E4 gene and E1 gene.

7. The method according to claim 5 or 6 wherein said functional fragment of said E4 gene comprises the open reading frame 6 of E4.

8. The method according to claim 1 wherein said recombinant adeno-associated virus further comprises a second selected gene operatively linked to regulatory sequences capable of directing expression of said second gene, said second gene capable of facilitating the conversion of said single stranded recombinant virus to its double stranded form upon expression, and capable of co-expression with said first selected gene in the target cell, whereby the second gene product is expressed in the target cell, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

9. The method according to claim 8 wherein the regulatory sequences directing expression of said second gene comprise an inducible promoter, and wherein expression of said second gene occurs in the presence of an inducing agent.

10. A recombinant adeno-associated virus comprising (a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing at least two selected genes or functional fragments thereof into a target cell in the absence of cell division; (b) a first selected gene operatively linked to regulatory sequences directing its expression, (c) a second selected gene operatively linked to regulatory sequences capable of directing expression of said second gene, said second gene capable of facilitating the conversion of said single stranded recombinant virus to its double stranded form upon expression, said first and said second genes flanked by the DNA of (a), said first and second gene capable of co-expression in the target cell.

11. The recombinant virus according to claim 10 wherein said second gene is selected from the group consisting of an adenovirus E4 gene, ORF6 of E4 and a functional fragment thereof.

12. The recombinant virus according to claim 10 wherein said second gene is the adenovirus E1 gene or a functional fragment thereof.

13. The recombinant virus according to claim 10 wherein the regulatory sequences directing expression of said second gene comprise an inducible promoter, and wherein expression of said second gene occurs in the presence of an inducing agent.

14. The recombinant virus according to claim 10 further comprising (d) an additional selected gene operatively linked to regulatory sequences capable of directing expression of said gene, said additional gene and said second gene capable of jointly facilitating the conversion of said single stranded recombinant virus to ~~its double stranded form upon expression of both said~~ second and additional genes, said first, second and additional genes flanked by the DNA of (a), and capable of co-expression in the target cell.

15. The recombinant virus according to claim 14 wherein said second gene is the adenovirus E4 gene or a functional fragment thereof and said additional gene is the adenovirus E1 gene or a functional fragment thereof.

16. The recombinant virus according to claim 15 wherein said functional fragment of E4 is the ORF6 sequence.

17. The recombinant virus according to claim 15 wherein the regulatory sequences directing expression of said additional gene comprise an inducible promoter, and wherein expression of said additional gene occurs in the presence of an inducing agent.

18. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell comprising the steps of: providing a recombinant adeno-associated virus of claim 10 through 16; infecting said target cells with said recombinant virus; and culturing said infected cells under conditions which enable expression of said selected genes in said target cell,

wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

19. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell comprising the steps of: providing a recombinant adeno-associated virus of claim 17; infecting said target cells with said recombinant virus; and contacting said target cell with an inducing agent which induces the expression of said second or additional gene in said target cell, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

20. A pharmaceutical composition comprising a recombinant adeno-associated virus and an agent which facilitates the conversion of said ss recombinant virus to its double stranded form in a target cell.

21. The composition according to claim 20 wherein said virus is selected from the group consisting of the recombinant viruses of claims 10 - 16.

22. The composition according to claim 20 wherein said virus is the recombinant virus of claim 17 and further comprising an inducing agent which induces the expression of said second or additional gene in said recombinant virus.

23. A mammalian cell transduced with the recombinant adeno-associated viruses of claims 10-17.

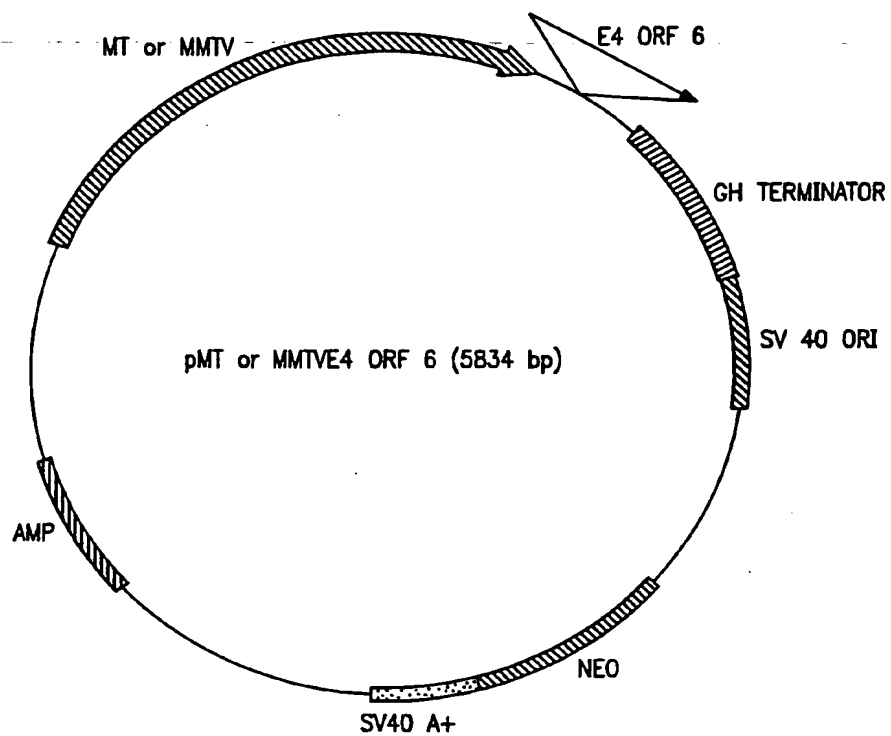


FIG. 1

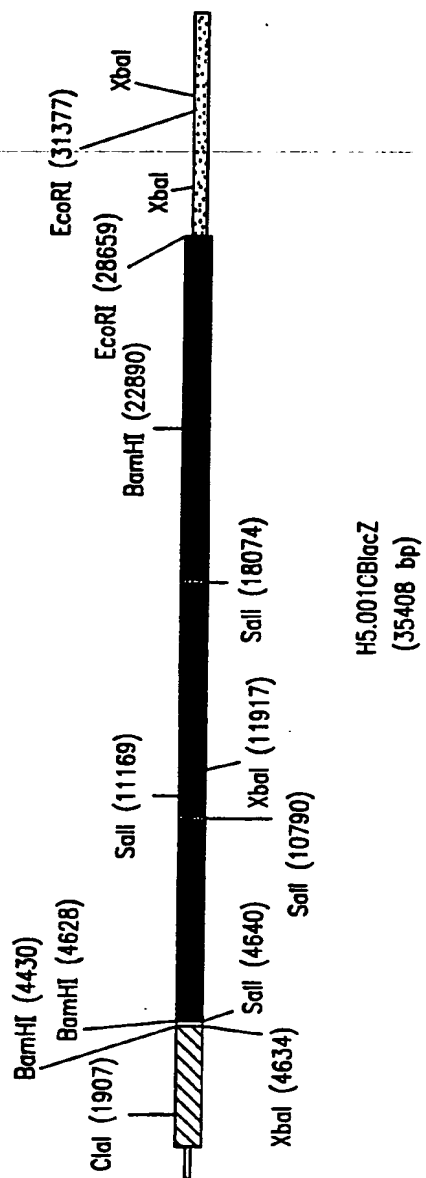
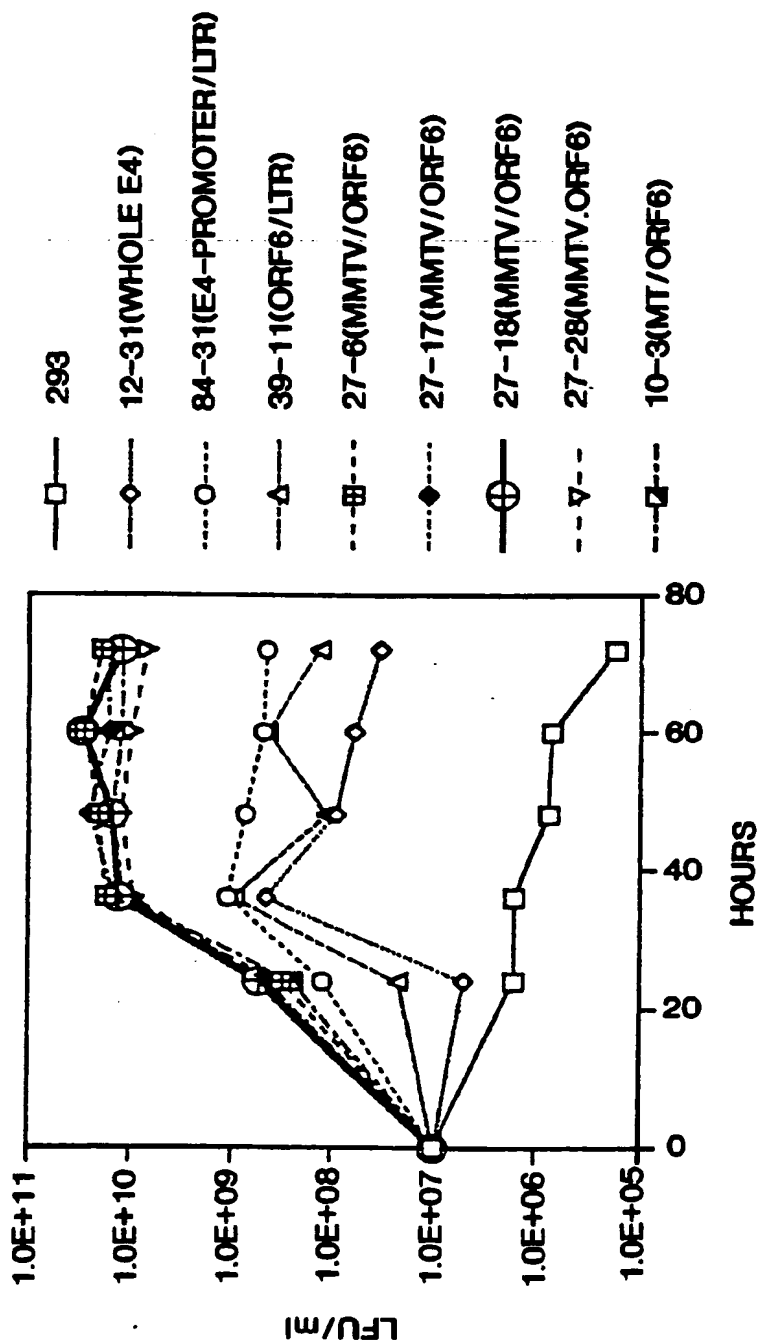


FIG. 2

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FIG. 3



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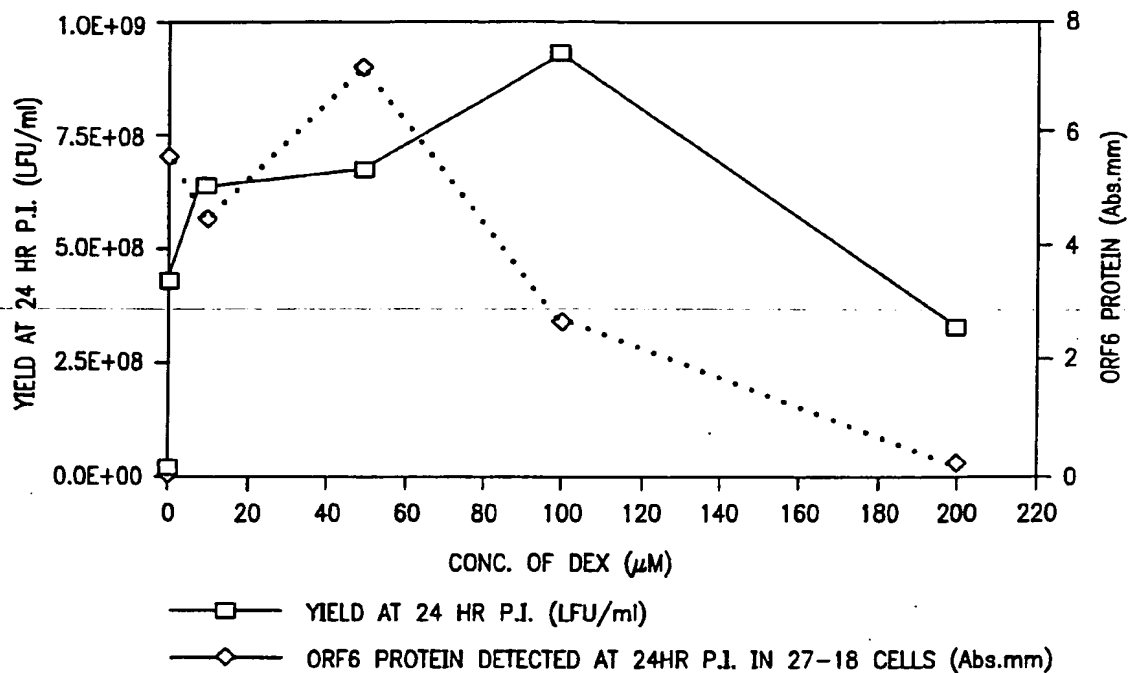


FIG. 4A

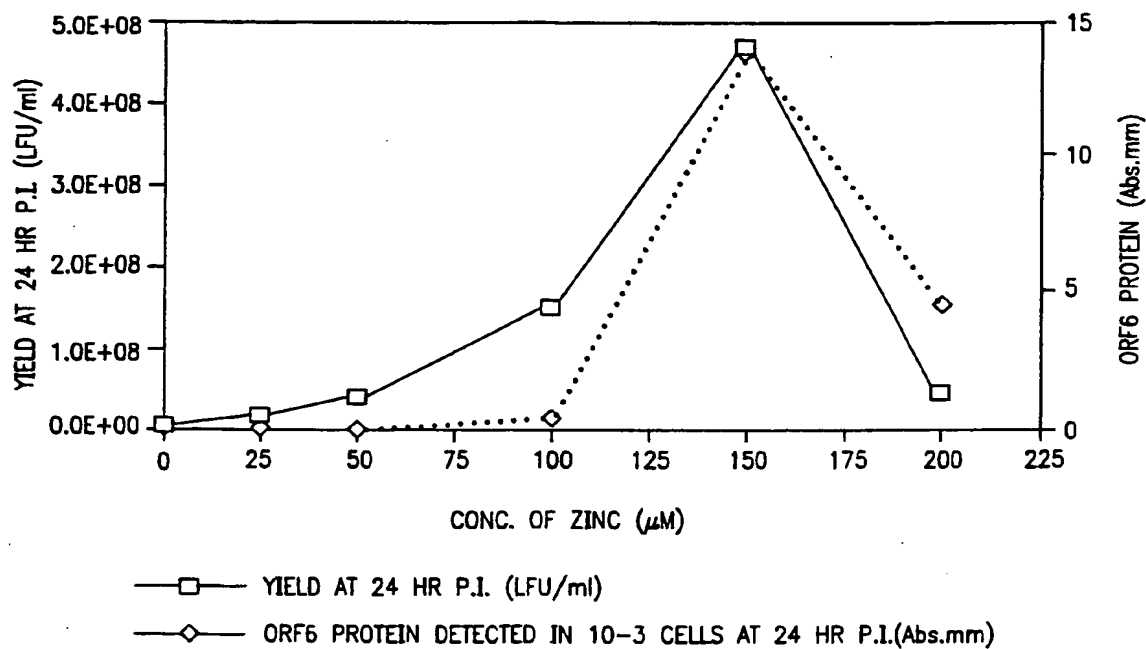


FIG. 4B

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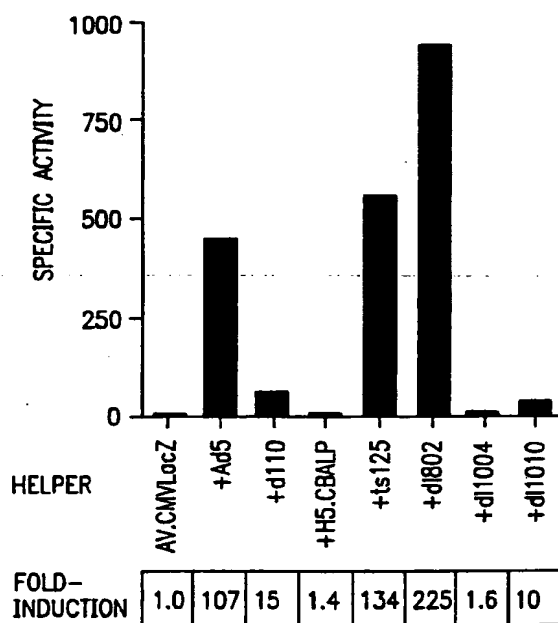


FIG. 5A

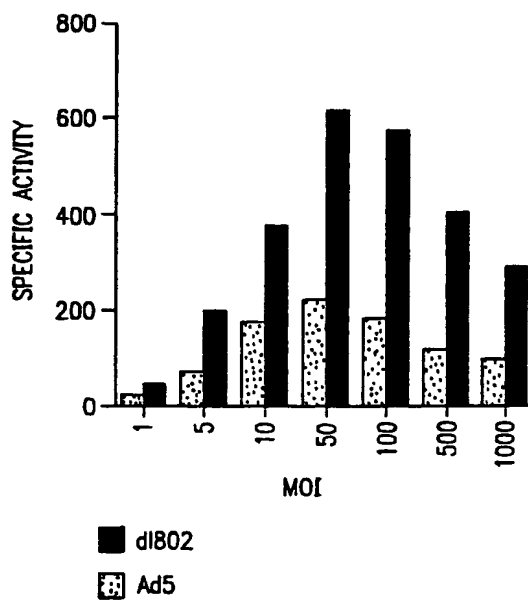


FIG. 5B

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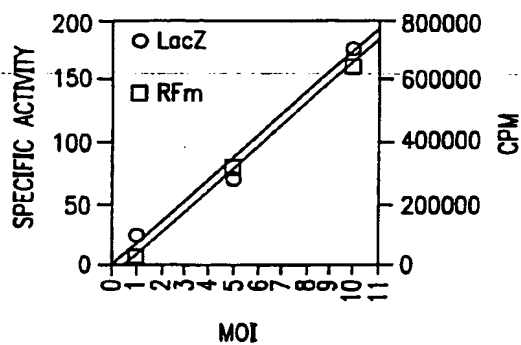


FIG. 6A

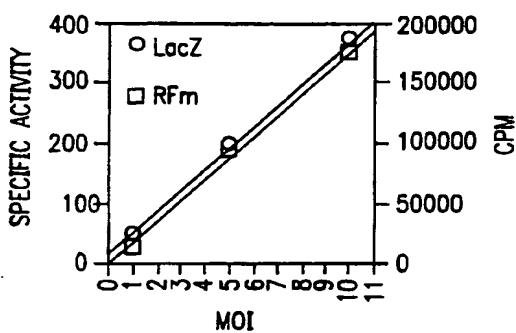


FIG. 6B

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FIG. 7A

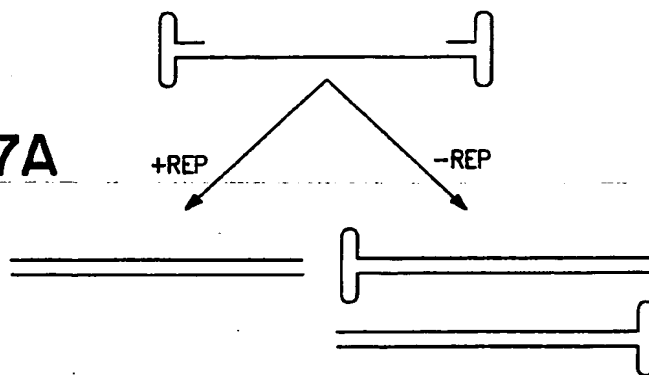


FIG. 7B

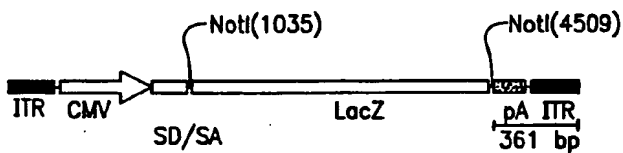
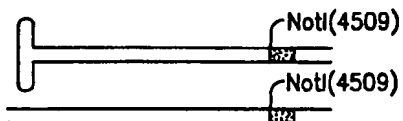


FIG. 7C



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FIG. 7D

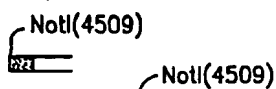
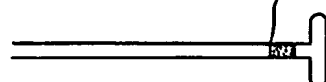


FIG. 7E



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FIG. 7F



FIG. 8A

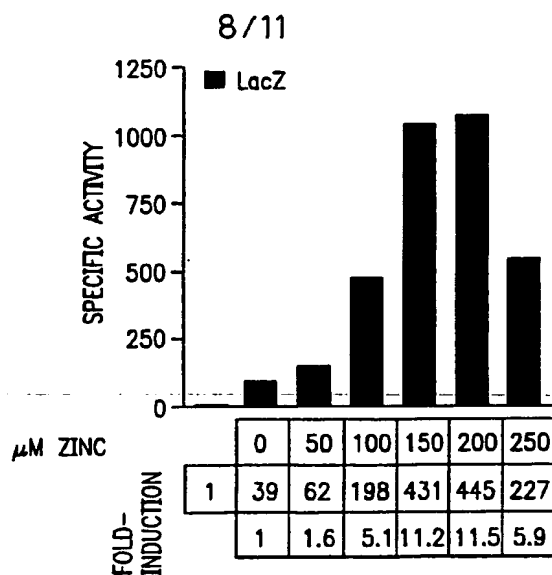


FIG. 8B

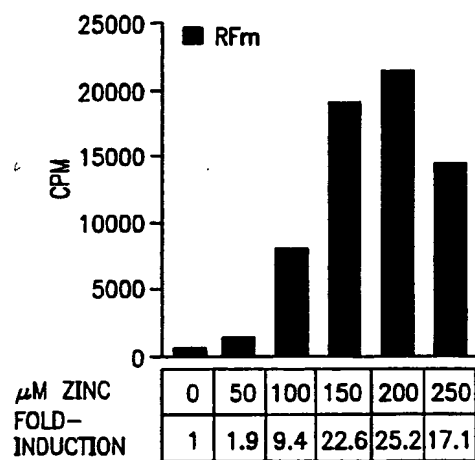
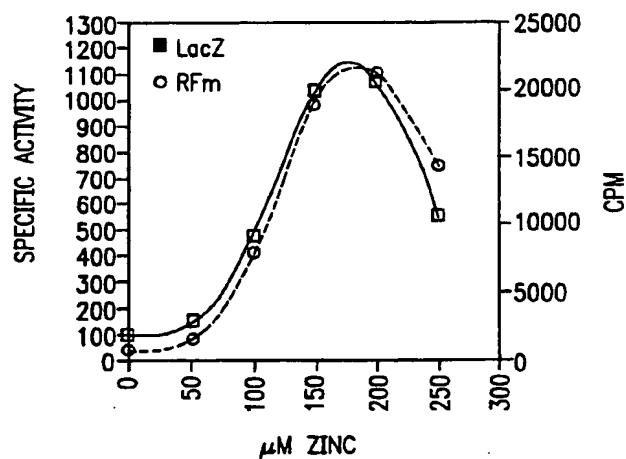


FIG. 8C



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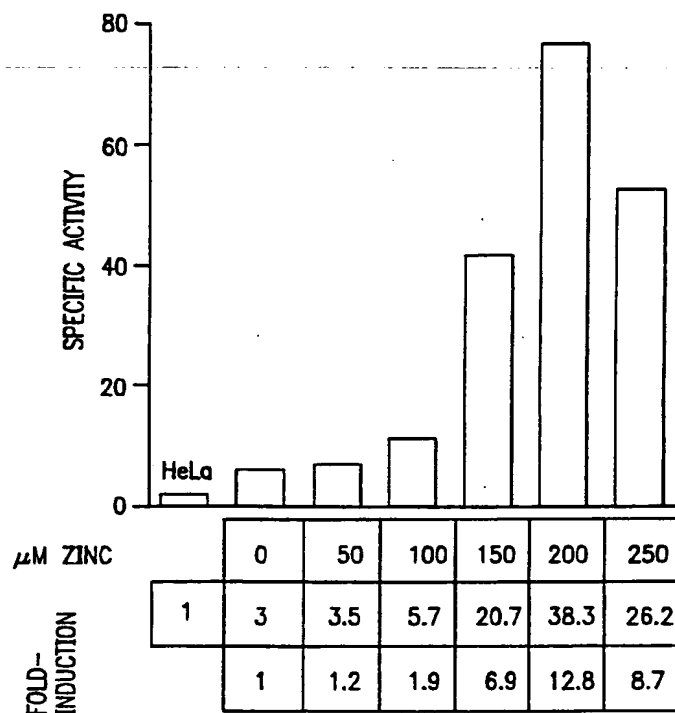


FIG. 9

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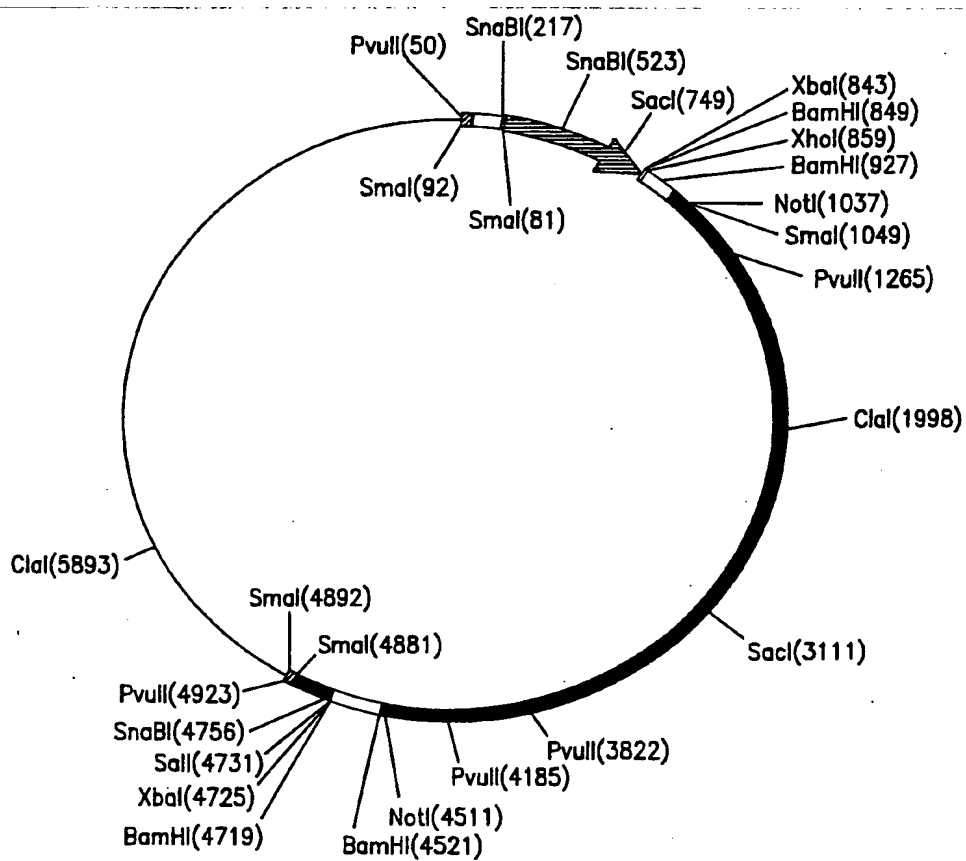


FIG. 10

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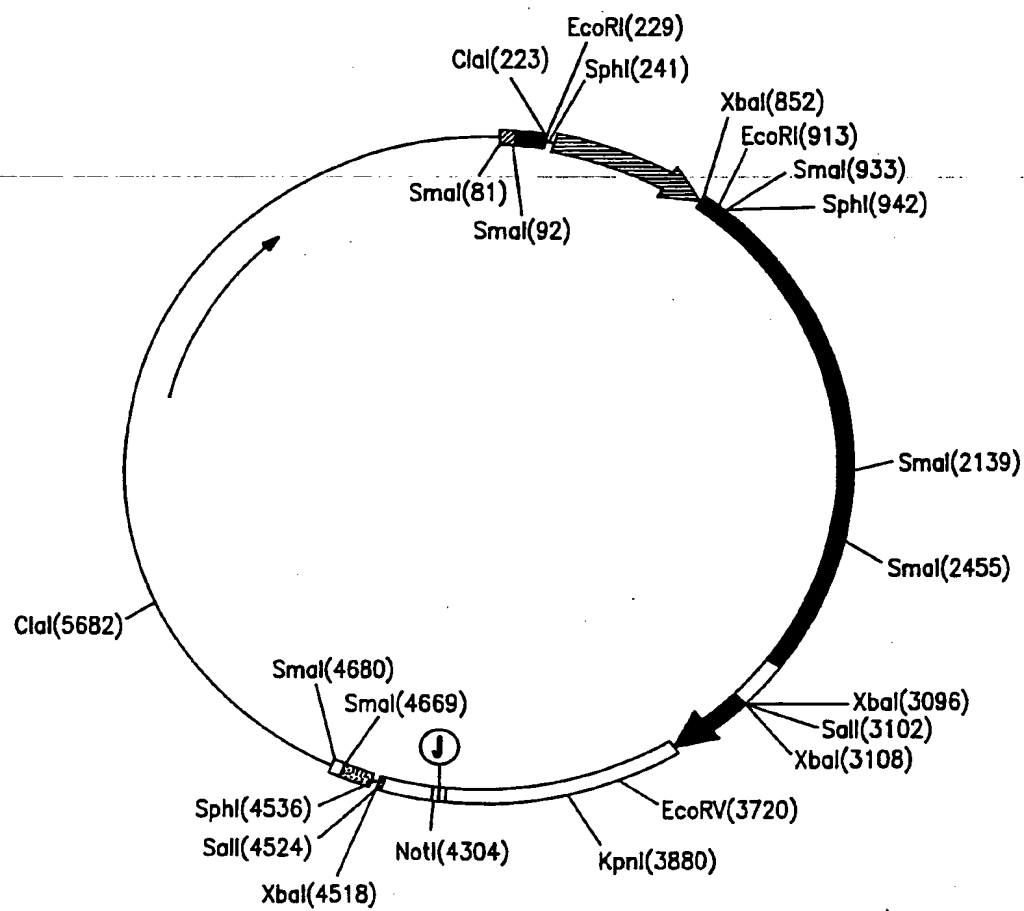


FIG. 11

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, 15/87, 5/10, A61K 35/76, 48/00		A3	(11) International Publication Number: WO 96/39530 (43) International Publication Date: 12 December 1996 (12.12.96)
(21) International Application Number: PCT/US96/10245 (22) International Filing Date: 4 June 1996 (04.06.96) (30) Priority Data: 08/462,014 5 June 1995 (05.06.95) US 08/549,489 27 October 1995 (27.10.95) US		Robindale Apartment F5, 1925 Lawrence Road, Havertown, PA 19083 (US). (74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US). (81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Applications or Grants (63) Related by Continuation US 08/462,014 (CIP) Filed on 5 June 1995 (05.06.95) US 08/549,489 (CIP) Filed on 27 October 1995 (27.10.95) (71) Applicant (for all designated States except US): THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; 133 South 36th Street, Philadelphia, PA 19104-3246 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). FISHER, Krishna, J. [US/US]; 4006 Pine Street, Philadelphia, PA 19104 (US). GAO, Guang-Ping [CN/US];		Published With international search report. (88) Date of publication of the international search report: 5 June 1997 (05.06.97)	
(54) Title: RECOMBIANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS, CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF			
(57) Abstract <p>An adenovirus E1/E4 expressing packaging cell line is provided, which permits the generation of recombinant adenoviruses deleted in both gene regions. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell is provided by infecting a target cell with a recombinant AAV comprising a selected transgene under the control of regulatory sequences. The infected cell is contacted with an agent which facilitates the conversion of single stranded recombinant virus to its double stranded form.</p>			

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INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/US 96/10245

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/86 C12N15/87 C12N5/10 A61K35/76 A61K48/00		
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